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"*IN VIVO*" AND "*IN VITRO*" EFFECT OF ASCORBIC ACID AND ACTH ON THE RAT ADRENAL CORTEX

BY A. DESMARAIS AND J. LEBLANC[1]

Abstract

Histochemical examination of adrenal glands of hypophysectomized rats given both ascorbic acid and ACTH showed an enlargement of the cortex and a decrease of sudanophilic substances, as compared to adrenals of hypophysectomized rats receiving ACTH alone. "In vitro" experiments on incubated slices of adrenal glands have shown that ascorbic acid and ACTH have a synergistic effect on the secretory activity of the cells of the adrenal cortex.

Introduction

It is now well established that adrenal ascorbic acid and cholesterol levels decrease when the gland is stimulated. For reference on this subject we refer to the extensive review of Sayers and Sayers (5). Dugal and Thérien (2) have shown that administration of ascrobic acid to rats and guinea pigs increases to a very great extent their resistance to cold. Studying the effects of ascorbic acid administration on the general adaptation syndrome, Dugal and associates (9) observed a faster decrease of the cholesterol level in animals exposed to cold. This was suggestive of some sort of a relation between ascorbic acid and the adrenocorticotropic hormone. More recently Thérien and Dugal (8) have shown that doses of ACTH, insufficient to maintain the weight of the adrenals of hypophysectomized rats, were very effective indeed when large doses of ascorbic acid (sodium ascorbate) were given simultaneously.

The present paper will give the results of the histochemical examination of the adrenal glands of the rats used in that last experiment (8) and also the results of a few experiments with incubated adrenal slices.

I. Effect of Ascorbic Acid and ACTH on the Adrenal Cortex of Hypophysectomized Rats

MATERIAL AND METHODS

Thirty-one hypophysectomized albino rats of the Wistar strain, weighing between 125 and 135 gm., were divided into three groups of equivalent weights.

[1] *Manuscript received August 7, 1951, and, as revised, September 25, 1951.*
Contribution from the Department of Experimental Physiology, Faculty of Medicine, Laval University, Quebec, Que.

All animals were fed ad libitum the diet described by Shaw and Greep (7). The left adrenal was removed on the 12th day after hypophysectomy, dissected free of surrounding fatty tissue, weighed, and fixed in neutral buffered 10% formalin for 48 hr. Four days after removal of the left adrenal, the following treatments were instituted:

Group Aa: 0.5 mgm. La-1-A equivalent ACTH Armour once a day by intraperitoneal injection + 150 mgm. sodium ascorbate divided into three doses given at 10 a.m., 12 p.m., and 4.30 p.m., also by intraperitoneal injection.

Group A: ACTH alone, same dose.

Group Ab: ACTH same dose + 70 mgm. sodium bicarbonate divided into three doses and administered the same way as sodium ascorbate.

These treatments were continued for 10 days, at which time the remaining adrenal was removed, weighed, and fixed in formalin in the same manner as the left one. In this way each rat was a control to itself, the left adrenal being used as control for the changes observed on the right after treatment.

After formalin injection, the adrenals were embedded in gelatin following the technique of Zwemer (11) and cut at 10 μ on the freezing microtome. On parallel sections were applied: the Sudan IV stain, the Schultz cholesterol test, and the Seligman (1) stain for the demonstration of ketosteroids. In all cases, the intensity of reaction was classified from zero to four and evaluated visually by two of us separately. Besides this, the outlines of the different zones of the cortex were reproduced with the camera lucida and subsequently measured as surfaces with a Keuffel and Esser compensating polar planimeter.

Results

1. *Histochemical Observations* (*Table I*)

(*a*) *Sudanophilia*

There was a general decrease in sudanophilia following all treatments. However, this decrease was significant in one group only, Group *Aa* receiving

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Fig. 1. Sudanophilia (Sudan IV) in left adrenal of hypophysectomized rat before treatment with sodium ascorbate and ACTH. Green filter. × 155.

Fig. 2. Sudanophilia (Sudan IV) in right adrenal of the same rat after above treatment. Green filter. × 155.

Fig. 3. Seligman reaction in adrenal slice of normal rat at 0 time incubation in K.-R. + ACTH and ascorbic acid. Red filter. × 155.

Fig. 4. Seligman reaction in parallel adrenal slice after 12 min. incubation in same medium as above. Red filter. × 155.

Fig. 5. Sudanophilia (Sudan black B). Normal adrenal. × 155.

Fig. 6. Sudanophilia (Sudan black B) in adrenal slice of adrenaline treated rat at 0 time incubation in K.-R. + ascorbic acid. × 155.

Fig. 7. Sudanophilia (Sudan black B) in parallel adrenal slice after 24 min. incubation in same medium as above. × 155.

Fig. 8. Seligman reaction. Normal adrenal. Red filter. × 155.

Fig. 9. Seligman reaction in adrenal slice of adrenaline treated rat at 0 time incubation in K.-R. + ascorbic acid. Red filter. × 155.

Fig. 10. Seligman reaction in parallel adrenal slice after 12 min. incubation in same medium as above. Red filter. × 155.

PLATE I

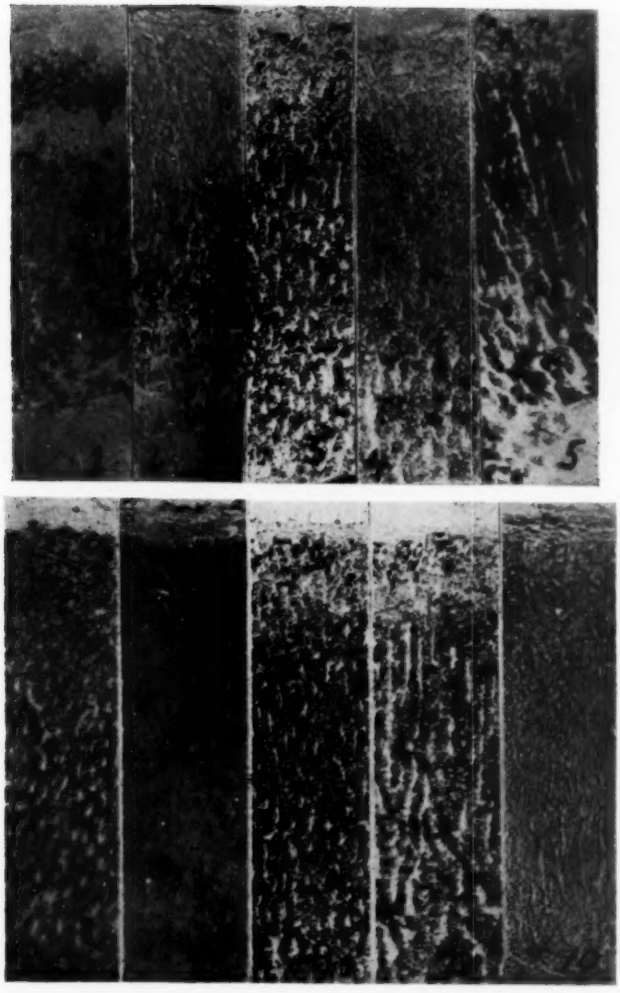

TABLE I

SUDANOPHILIA. PER CENT DECREASE IN SUDANOPHILIA (SUDAN IV) FROM LEFT TO RIGHT ADRENAL OF HYPOPHYSECTOMIZED RATS GIVEN THE TREATMENTS DESCRIBED IN PAGES

Group	Decrease glomerulosa, %	"t" value	Decrease fasciculata, %	"t" value
Aa	−65. ± 11.0*	*4.28*	−56. ± 8.2	*4.62*
A	−28. ± 7.5	1.08	−22 ± 7.1	1.11
Ab	−25. ± 9.2	2.02	−21. ± 4.3	2.14

*Standard error: $\sqrt{\frac{\Sigma(d)^2.}{n\,(n\text{-}1)}}$.

both ACTH and sodium ascorbate. Examples of these changes can be seen in Figs. 1 and 2.

(*b*) *Schultz Cholesterol Test*

This test was always parallel with Sudan IV.

(*c*) *Seligman Reaction*

We observe a general increase in this reaction in all groups, with no difference from one group to the others.

2. *Section Surface Measurements* (*Table II*)

TABLE II

EFFECT OF TREATMENT ON THE SECTION SURFACE OF THE ADRENAL CORTEX OF HYPOPHYSECTOMIZED RATS

Group	Measurements on:	Left adrenal	Right adrenal	% diff.	*t*
Aa	Glomerulosa	8.1 ± 0.58*	10.9 ± 1.00	+24.5	*2.41*
	Fasciculata	21.2 ± 1.69	26.4 ± 3.99	+24.5	1.21
	Whole cortex	28.6 ± 1.39	35.6 ± 2.97	+24.4	*2.14*
A	Glomerulosa	11.2 ± 0.55	10.1 ± 0.77	− 9.8	1.17
	Fasciculata	22.9 ± 1.46	19.1 ± 1.51	−16.6	1.81
	Whole cortex	33.4 ± 1.53	30.3 ± 1.68	− 9.2	1.36
Ab	Glomerulosa	9.2 ± 0.50	9.7 ± 0.63	+ 5.4	0.62
	Fasciculata	23.3 ± 1.44	20.1 ± 1.59	−13.7	1.49
	Whole cortex	32.5 ± 1.57	28.9 ± 1.54	−11.1	1.64

*Standard error: $\sqrt{\frac{\Sigma\,(d)^2}{n(n\text{-}1)}}$.

In Groups *A* and *Ab*, there was a general decrease in section surface. In Group *Aa*, treated with both ACTH and sodium ascorbate, a significant increase in surface section was observed for the glomerulosa and the whole cortex.

II. Effect of Ascorbic Acid and ACTH on Incubated Adrenal Slices

Material and Methods

All rats used were males of the Harvard strain, weighing between 100 and 125 gm., either normal or in a state of alarm reaction following adrenaline administration. In the alarm reaction experiments, adrenaline was administered to fasting animals in two subcutaneous injections of the Parke-Davis solution (1:100 diluted with physiological saline), at 0 and the 12th hour of the fast, and the adrenals were removed at the 24th hour. Adrenaline was administered at a dosage level of 1.5 γ per gram body weight. Upon removal of the adrenals, the left gland was immediately fixed in 10% neutral formol and the other gland was frozen and cut into slices for incubation. The slices were then incubated in an atmosphere of pure oxygen in either of the following media:

1. Krebs-Ringer phosphate (K.-R.) pH 7.2 alone;
2. K.-R. containing 100 γ of ascorbic acid per milliliter;
3. K.-R. plus 200 γ La-1-A equivalent of ACTH per 3 ml.;
4. K.-R. plus both ascorbic acid and ACTH.

Three milliliters of incubation medium were used in all experiments. Slices from the same adrenal were removed singly from the incubation vessel after 12, 24, 36, 48, and 60 min. of incubation. After removal, the slices were immediately fixed in 10% neutral formol for 48 hr. Sections of 10 μ thickness were cut from the fixed slices as well as from the control left adrenal and studied by the following techniques: Sudan black B, Seligman (1) reaction, Schultz cholesterol test, and birefringence.

Results (Table III)

1. Normal Rats

(*a*) *Sudan Black B*

No appreciable change was observed with either treatment.

(*b*) *Seligman Reaction* (S.R.)

The most striking result was the almost complete disappearance of S.R. positive material in slices incubated with both ascorbic acid and ACTH. See Figs. 3 and 4.

2. Adrenaline Treated Rats

In these slices, we noted a disappearance of both sudanophilic and S.R. positive substances in slices incubated with ascorbic acid. See Figs. 5 to 10.

3. Schultz Cholesterol and Birefringence Tests

None of these two tests did undergo any appreciable change which could be linked to the previous observations.

TABLE III

CHANGES IN SUDANOPHILIA (SUDAN BLACK B) AND SELIGMAN REACTION (S.R.) DURING INCUBATION OF ADRENAL SLICES

	Time of incubation (min.)					
	0	12	24	36	48	60
Normal rats (three runs)						
Sudan						
K.-R. alone	4+	4+	3+	4+	4+	5+
K.-R. + asc. acid	4+	5+	5+	5+	4+	4+
K.-R. + ACTH	4+	4+	4+	3+	3+	4+
K.-R. + asc. acid and ACTH	4+	4+	5+	4+	5+	4+
S.R.						
K.-R. alone	2+	3+	2+	3+	2+	3+
K.-R. + asc. acid	2+	4+	3+	2+	4+	3+
K.-R. + ACTH	2+	2+	1+	2+	1+	2+
K.-R. asc. acid and ACTH	2+	0	0	0	0	1+
Adrenaline treated rats (three runs)						
Sudan						
K.-R. alone	1+	3+	2+	1+	1+	0
K.-R. + asc. acid	1+	2+	0	0	0	0
S.R.						
K.-R. alone	1+	3+	3+	3+	2+	3+
K.-R. + asc. acid	1+	0	0	0	0	0

Conclusion and Discussion

We have seen that in hypophysectomized animals, ascorbic acid had a potentiating effect on the action of ACTH as measured by enlargement of the cortex and a decrease in sudanophilia (Sudan IV). This decrease in sudanophilia can be interpreted in terms of cholesterol since the Schultz test, performed on parallel slices, always showed similar changes. As a decrease in cholesterol is generally (3, 4, 5, 6, 10) interpreted in terms of adrenal cortex activation, it is therefore legitimate to say that ascorbic acid enhances the effect of ACTH on the adrenal cortex.

Similar results were obtained with incubated slices. In glands from normal animals, both ascorbic acid and ACTH must be present to cause a maximum activation of the adrenal cortex as measured by the Seligman reaction; in glands from animals pretreated with adrenaline, (glands already stimulated by endogeneous ACTH), the presence of ascorbic acid is still needed to obtain a maximum activation, as measured by both the Seligman reaction and sudanophilia.

We have, at the present time, no explanation for the fact that, in incubated slices, no changes in cholesterol could be recorded with either the Schultz test

or birefringence. Truly, the lack of sensitivity of these tests does not permit one to conclude that no changes in cholesterol occurred. On the contrary, the changes in sudanophilia would suggest variations in cholesterol. It might be that such changes were too small to be recorded visually.

We have seen that, *in vivo*, while sudanophilia is decreased in the adrenals of hypophysectomized rats given ascorbic acid and ACTH, there is a strong indication that the Seligman reaction is increased. We found that, *in vitro*, sudanophilia and Seligman reaction decreased simultaneously; it might be that in isolated glands, free from circulating blood, the supply of precursors is cut off and both precursors and end products decrease at the same time.

Acknowledgments

The first part of this work was made possible through a grant of the Defence Research Board of Canada; the second part was done by one of us, A.D., while on a Rockefeller Fellowship at the Biological laboratories, Harvard University, under the direction of Prof. Alden B. Dawson. The ACTH was generously supplied by the Armour Laboratories.

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THE CONCENTRATION AND ACCUMULATION OF LIPIDS IN THE TUMOR COMPONENT OF A TUMOR–HOST ORGANISM, WALKER CARCINOMA 256 IN ALBINO RATS[1]

By Eldon M. Boyd and H. D. McEwen

Abstract

A study was made of the lipid concentration and accumulation in the tumor component of a tumor–host organism at progressive intervals during its growth using Walker Carcinoma 256 in albino rats. At the end of each of 7, 14, 21, and 28 days of tumor growth, and covering the entire life history of Walker Carcinoma 256, 10 tumor–rat organisms were sacrificed and appropriate sections of the tumor removed for differential lipid analysis by oxidative micromethods. The outstanding feature revealed in these studies was the maintenance, throughout its life history, of high concentrations of phospholipid, free and esterified cholesterol in Walker Carcinoma 256, and the increasing accumulation of large amounts of these lipids in the tumor component of the tumor–host organism. There was no marked accumulation of neutral fat which, in the tumor, declined to almost 1/10th its initial concentration concurrently with the visible loss of storage fat in the host component. A day or two before death, the central core of the tumor appeared necrotic, its concentration of phospholipid declined and its concentration of free cholesterol rose. Otherwise, the lipid composition of the tumor was uniform throughout the cross section.

Introduction

The relationship between a tumor and its host might be likened to that of two animals living out together the remainder of their life span, locked in one room and sharing, however unequally, one source of food and one avenue for disposal of waste products. While this simile is not entirely apt, it serves to emphasize the concept of tumor metabolism as an integral part of the metabolism of a dual tumor–host organism rather than as a separate entity. Under such circumstances, one might attempt to evaluate the metabolic relation between tumor and host from data upon the chemical composition of each, made available at intervals from the beginning to the end of their period of propinquity. If, for this purpose, one were to select a tumor and host, such that the mass of one relative to the mass of the other changed markedly during the interval of their association, then metabolic differences, measured by static quantitative chemical analysis, should be enhanced by the simple process of comparing figures obtained by multiplying mass by percentage composition.

With this objective in view and with lipid metabolism specifically in mind, the lipid composition of Walker Carcinoma 256 was determined at regular and progressive intervals during its period of growth. Following subcutaneous inoculation of sections of this tumor into young albino rats, growth of the carcinoma is rapid and at death one month later tumor weight approximates one-half the weight of the residual carcass of the host. Toward the end of this time, the host rat becomes increasingly more inactive, emaciated, and cachectic. In the initial investigation to be reported below, attention was

[1] *Manuscript received December 12, 1951.*
A contribution from the Department of Pharmacology, Queen's University, Kingston, Ont.

confined to a study of the accumulation of lipids in the tumor. These were removed from groups of albino rats at the end of weekly intervals of growth up to the end of the fourth week and analyzed, by oxidative micromethods, for total and component lipids. The results demonstrated that relatively large amounts of lipids, mostly phospholipid, accumulate in Walker Carcinoma 256 in the terminal phases of its growth in albino rats.

The presence of a high concentration of phospholipid in Walker Carcinoma 256, in terms of grams per 100 gm. of tissue, was reported by Haven (5) who analyzed tumors taken from host rats at or near death and who has reviewed earlier data upon the lipid composition of experimental tumors. More recently, Mider, Sherman, and Morton (7) reported that the total lipid of the combined tumor and residual carcass at death of rats bearing Walker Carcinoma 256 was less than that of tumor-free controls.

Methods

The Walker Carcinoma 256 used in this research was obtained in 1947 by one of us (H. D. McE.) from Dr. F. L. Haven of the University of Rochester, N.Y., who had maintained the tumor from a sample received from the Institute of Cancer Research of Columbia University some 12 years earlier (5). It has retained its major original characteristics. In four years of transplanting at Queen's University, the activity of the tumor has increased somewhat. Very few inoculations fail to develop into tumors and tumor growth results in death of the host rat within 30 days. In Haven's (5) studies, tumors were removed from 20 albino rats after an average of what may be calculated to have been 36 days of growth. In the experiments of Mider, Sherman, and Morton on Walker Carcinoma 256 reported in 1949 (7), the average weight of 25 tumors removed at or near death of the host rat may be calculated from their published data to have been 55 gm. After 28 days of growth, and at or near death of the host rat, the average weight of tumors removed from our rats was 63.2 gm. with a standard deviation of 23.0 gm.

Using aseptic precautions, sections of 20 to 30 mgm. of tissue were removed from the periphery of an actively growing tumor and implanted subcutaneously, with the aid of a trocar, into the inguinal region of healthy, young, male albino rats which had a mean body weight of 82.9 gm., standard deviation 14.0 gm., on the day of inoculation. The albino rats were from a colony which has been maintained in the animal quarters of this department at Queen's University since 1937. They were fed Purina Fox Chow Checkers and water ad libitum. Data reported in this communication were obtained on tumors removed from 50 such tumor-bearing rats.

Ten tumor–host organisms were sacrificed, by fracture of the cervical spinal column, at the end of each of one, two, three, and four weeks of tumor growth. The partition of tissue weight in these tumor–host organisms was determined in each instance by weighing in grams the tumor–host organism, the separated tumor, and the residual host component. These values have been summarized in Table I.

TABLE I

THE WEIGHT PARTITION OF THE TUMOR–HOST ORGANISM, WALKER CARCINOMA 256 IN ALBINO RATS. (VALUES ARE EXPRESSED AS GRAMS)

Weight	Calculation	Weeks of tumor growth			
		1	2	3	4
Tumor plus host	Mean	117.3	152.7	192.9	206.0
	Standard deviation	18.2	10.8	24.8	32.7
Tumor	Mean	0.8	16.4	41.8	63.2
	Standard deviation	0.4	3.5	15.9	23.0
Host	Mean	116.5	136.3	151.1	142.8
	Standard deviation	19.1	15.2	17.3	40.7

The water content of the tumor component was found to be high and uniform throughout growth, as may be seen from the values summarized in Table II. Because the whole of the small tumors removed after one week of growth was required for lipid analysis, water content was determined at this interval upon tumors removed from 10 litter-mate animals.

TABLE II

THE WATER CONTENT OF WALKER CARCINOMA 256 AT PROGRESSIVE STAGES OF ITS GROWTH (VALUES ARE EXPRESSED AS GRAMS OF WATER PER 100 GM. OF TUMOR)

Weeks of growth	Water content			
	Body of tumor		Central core	
	Mean	Standard deviation	Mean	Standard deviation
1*	85.1	0.94	85.1	0.94
2	85.1	0.84	84.9	1.23
3	85.1	1.05	85.0	2.41
4	85.1	0.17	85.4	0.20

**Data in this line were obtained upon the whole, indivisible tumor.*

Preliminary trials indicated that 1 gm. specimens of the tumor contained amounts of lipids within the range of amounts which could be determined with maximum efficiency (optimal recovery and lowest analytical variation) by the oxidative micromethods employed. Specimens of approximately double this weight were removed from the body ("periphery") and central core of tumors at the end of two, three, and four weeks of growth and, as noted above, the whole tumor was used as the specimen at the end of one week's growth. Half the specimen was used for determination of water content and the remaining half was cut into small pieces, triturated with purified sand in a mortar, extracted with alcohol–ether and analyzed for total lipid, neutral fat, total

fatty acids, total cholesterol, ester cholesterol, free cholesterol, and phospholipids by a differential oxidative technique previously described (3). The results obtained were expressed as grams of lipid per 100 gm. dry weight of tumor.

It should be noted that in this system of lipid analysis, neutral fat is calculated by subtracting from total fatty acids the sum of phospholipid fatty acids and cholesterol ester fatty acids, and expressing the difference as a triglyceride. It is possible that in Walker Carcinoma 256, such residual fatty acids may be present in a form other than triglyceride fatty acid esters.

Results

The data upon lipid composition of Walker Carcinoma 256 were assembled in tables, each of which contained the values determined at the end of one, two, three, and four weeks of tumor growth respectively. Each table contained seven columns, corresponding to the seven lipids estimated, and each column contained 10 entries corresponding to the 10 tumors analyzed at the end of each week of tumor growth. The mean and standard deviation of the 10 entries were calculated after Bradford Hill (6) and these latter values have been asembled in Tables III and IV, Table III containing values obtained from analyses of the body of Walker Carcinoma 256 and Table IV values from analysis of the central core of the tumor.

TABLE III

THE LIPID COMPOSITION OF THE BODY OF WALKER CARCINOMA 256 AT PROGRESSIVE STAGES DURING ITS GROWTH

(Values are expressed as grams of lipid per 100 gm. dry weight of tumor)

Lipid	Calculation	Weeks of tumor growth			
		1*	2	3	4
Total lipid	Mean	28.06	14.83	13.93	14.22
Total lipid	Standard deviation	10.54	2.31	4.47	2.91
Neutral fat	Mean	18.95	5.36	3.58	2.78
Neutral fat	Standard deviation	9.59	2.42	2.27	2.94
Total fatty acids	Mean	22.74	10.47	8.79	8.77
Total fatty acids	Standard deviation	9.52	2.06	2.76	2.24
Total cholesterol	Mean	2.25	2.00	2.68	2.26
Total cholesterol	Standard deviation	1.86	0.49	1.97	0.91
Ester cholesterol	Mean	1.03	0.81	1.21	0.92
Ester cholesterol	Standard deviation	0.85	0.38	1.52	0.72
Free cholesterol	Mean	1.22	1.19	1.47	1.34
Free cholesterol	Standard deviation	0.69	0.23	0.79	0.28
Phospholipid	Mean	6.18	6.93	6.85	8.57
Phospholipid	Standard deviation	1.19	1.29	1.71	2.79

**Data in this column represent analyses on the whole tumor.*

TABLE IV

THE LIPID COMPOSITION OF THE CENTRAL CORE OF WALKER CARCINOMA 256 AT PROGRESSIVE STAGES DURING ITS GROWTH

(Values are expressed as grams of lipid per 100 gm. dry weight of tissue)

Lipid	Calculation	Weeks of tumor growth			
		1*	2	3	4
Total lipid	Mean	28.06	13.83	17.43	13.26
Total lipid	Standard deviation	10.54	1.98	5.49	3.20
Neutral fat	Mean	18.95	4.61	6.78	5.51
Neutral fat	Standard deviation	9.59	2.41	5.10	3.79
Total fatty acids	Mean	22.74	9.09	11.54	8.64
Total fatty acids	Standard deviation	9.52	1.88	3.75	3.04
Total cholesterol	Mean	2.25	2.46	3.41	2.99
Total cholesterol	Standard deviation	1.86	0.13	1.77	0.81
Ester cholesterol	Mean	1.03	0.87	1.21	1.06
Ester cholesterol	Standard deviation	0.85	0.37	1.61	0.47
Free cholesterol	Mean	1.22	1.59	2.20	1.93
Free cholesterol	Standard deviation	0.69	0.24	0.66	0.57
Phospholipid	Mean	6.18	6.17	6.40	4.04
Phospholipid	Standard deviation	1.19	0.79	3.51	1.50

**Data in this column represent analyses on the whole tumor.*

TABLE V

THE ACCUMULATION OF LIPIDS IN THE TUMOR COMPONENT OF THE WALKER CARCINOMA 256 – ALBINO RAT ORGANISM

(The results are expressed as grams of tumor lipid per kilogram of tumor–host organism)

Lipid	Weeks of tumor growth			
	1	2	3	4
Total lipid	0.28	2.38	4.51	6.50
Neutral fat	0.19	0.86	1.16	1.27
Total fatty acids	0.23	1.68	2.84	4.00
Total cholesterol	0.022	0.32	0.87	1.03
Ester cholesterol	0.010	0.13	0.39	0.42
Free cholesterol	0.012	0.19	0.48	0.61
Phospholipid	0.062	1.11	2.21	3.92

1. Total Lipid

The concentration of total lipid was found to be highest at the end of one week of tumor growth. Between the end of the first week and the end of the second week, the concentration of total lipid fell to approximately one-half its former value in both the body and central core of the tumor. The mean at

the end of one week (28.06) minus the mean in the body at the end of two weeks (14.83) yielded a mean difference of 13.23 which was calculated to have a standard error of 3.42, indicating statistically that there were more than 99 chances out of 100 that the two samples came from different universes. Similarly, the mean difference between total lipid values in the central core of the tumor at the end of two weeks and those at the end of one week was calculated to be 14.23 with a standard error of 3.39, indicating statistically that it was even more unlikely that these two samples were drawn from the same universe.

This drop in the level of total lipid could reflect an alteration in lipid metabolism of the tumor or of the host or both. The maintenance of the reduced total lipid concentration in Walker Carcinoma 256 at or near the same level during the second, third, and terminal fourth weeks of existence, in spite of an obvious loss of increasing amounts of fat by the host, suggests that this may represent a minimal tolerated concentration, the demands for maintaining which receive top priority in the tumor–host organism and are met by increasing inroads upon lipids or lipid precursors supplied to, or obtained from, the host fraction of the tumor–host organism.

2. Neutral Fat

As may be seen from the data in Tables III and IV, the early drop in concentration of total lipid of some 14 gm. per 100 gm. dry weight of tumor was due to a drop in concentration of neutral fat which averaged also some 14 gm. This decrease occurred between the 7th and 14th days of tumor growth and to essentially the same extent in the body and central core of the tumor, the mean differences being over four times the standard errors of the mean differences.

Following this initial sharp drop in the concentration of neutral fat, the lipid continued to decline slowly in value in the body of the tumor between the 14th and terminal 28th day, the mean difference of 2.58 gm. per 100 gm. dry weight being just over twice its standard error. In the body of the tumor, the concentration of neutral fat had fallen thus, from 18.95 gm. per 100 gm. on the seventh day to 2.78 gm. per 100 gm. on the 28th or terminal day. Neutral fat in the central core of the tumor was prevented from falling in concentration during the latter half of the tumor life history, its terminal concentration being 5.51 gm. per 100 gm. dry weight. As will be shown below, release of fatty acids from necrotic hydrolysis of phospholipids could have accounted for maintenance of neutral fat levels of the order of 5% in the central core.

3. Total Fatty Acids

Progressive changes in the concentration of tumor total fatty acids paralleled those of total lipid and of neutral fat. Between the 7th and 14th days, the concentration of total fatty acids decreased by over 50%. Thereafter in the body of the tumor the mean level continued to fall slightly but not sufficiently to be proved statistically significant with the number of samples

available. There was no appreciable change in total fatty acid concentration in the central core between the 14th and 28th days.

4. Total Cholesterol

In contrast to the above changes in concentration of total lipid, neutral fat, and total fatty acids during progressive growth of Walker Carcinoma 256, there was at no time any statistically significant alteration in the concentration of total cholesterol in any part of the tumor. After three weeks of tumor growth, mean values for total cholesterol rose in the central necrotic core; the mean increase was not sufficient in itself to be proved statistically significant, but was due to an increase in the concentration of free cholesterol which was significant, as will be shown below. The average concentration of total cholesterol at all stages of growth was of the order of 2 to 3 gm. per 100 gm. dry weight of tissue. The results suggest that this is a minimal critical level maintained at all costs by the tumor–host organism.

5. Ester Cholesterol

There were also no significant changes at any time and in any part of the tumor in the concentration of ester cholesterol which was maintained at a level of some 1 gm. per 100 gm. dry weight throughout. Note the absence of any increased concentrations of ester cholesterol in the necrotic core of the four-week-old tumors, Table IV. In nonmalignant body tissues, retrograde change may be accompanied by an increase in the concentration of ester cholesterol, usually at the expense of free cholesterol (1), although this is not always the case (4). Haven (5), analyzing 27- to 52-day-old Walker Carcinoma 256, reported lower concentrations of ester cholesterol in the peripheral portions and higher concentrations in the central portions than was found herein for the body and central core; otherwise her values correspond closely to those for 28-day-old tumors assembled in Tables III and IV. Assuming the central necrotic core to consist of dead tissue at 28 days, one might expect a gradual hydrolysis of cholesterol esters (2). In spite of these a priori assumptions, our results indicate that during the relatively short, rapid growth of Walker Carcinoma 256 in albino rats of our colony, the concentration of ester cholesterol is maintained at a critical minimal level of about 1% dry weight.

6. Free Cholesterol

There was at no time any significant change in the concentration of free cholesterol in the body of Walker Carcinoma 256. The average concentration was slightly over 1 gm. per 100 gm. dry weight which could be regarded as a minimal critical level for this tissue.

The initial concentration after two weeks of growth was also of this order in the central core of the tumor but increased as the core became necrotic in the last two weeks of growth. The maximal increase in the core averaged 0.98 gm. per 100 gm. dry weight, which represented an increase over initial concentration of 80%, the mean increase having a standard error of 0.32 gm. per 100 gm. The accumulation of free cholesterol in the necrotic core could

not have been due to hydrolysis of cholesterol esters in this area of the tumor and must have been due either to local synthesis, which seems unlikely, or to the transportation to, and precipitation in, the necrotic core of free cholesterol from the body of the tumor or from the host. A consideration of the figures listed in Tables III and IV suggests that the extra free cholesterol which was stored in the necrotic core of the tumor, came from the host.

7. Phospholipid

From the data collected in Tables III and IV, it is apparent that for three-quarters of the period of growth of Walker Carcinoma 256 in our laboratories at Queen's University, phospholipid concentration was maintained at a level of some 6 to 7 gm. per 100 gm. dry weight, in all parts of the tumor. During the last week of growth, the mean phospholipid concentration increased by about one-third in the body of the tumor and decreased by about one-third in the necrotic core. This mean increase in tumor body phospholipid was of equivocal statistical significance, since it was calculated to be just over or just under twice its standard error, depending upon which previous mean was used for comparison. The mean decrease in tumor core phospholipid was calculated, in a similar manner, to be from two to four times its standard error.

The mean increase in tumor body phospholipid between 21 and 28 days was 1.72 gm. per 100 gm. which may be estimated to have been composed of 1.15 gm. of fatty acid. At the same time there was a mean loss of 0.80 gm. of neutral fat. Hence, the mean increase in tumor body phospholipid could have been due almost entirely to tumor synthesis of phospholipid from fatty acids released in the neutral fat fraction. There is some indication, from the figures recorded in Tables III and IV, concerning the fate of the phospholipid which was broken down in the necrotic tumor core; the released fatty acids may have separated by analysis into the neutral fat fraction, the concentration of which did not decline in the core as it did in the body of the tumor.

The concentration of phospholipid in Walker Carcinoma 256 was higher than is found in rat tissue generally. Throughout most of the life of the tumor, phospholipid accounted for half the tumor lipids, also a higher proportion than is found in rat tissues generally. In nontumor tissues, the concentration of phospholipid is generally greater with greater physiological activity (1, 4). This seems to be true for malignant tissues as well (5). These results further reflect the tremendous activity of Walker Carcinoma 256.

Discussion

As previously stated, this investigation was started with the idea of studying progressive alterations in lipid metabolism in the Walker Carcinoma – albino rat organism. The initial phase of the investigation was concerned with progressive changes in the lipid concentration of the tumor and the results, described above, may be reviewed by stages of growth.

After seven days of growth, the tumor was less than 1% of body weight and had little visible effect upon the albino rat host. Its lipid content was similar to that of some normal rat tissues and organs, making up one-quarter

to one-third of the dry weight and being composed mostly of neutral or storage fat but with a higher than average concentration of phospholipid, cholesterol, and cholesterol esters.

Between the 7th and 14th days of growth, the tumor had attained 10%, or over, the weight of the tumor–host organism. By this time there was evidence of marked alteration in its lipid metabolism which was manifested by a decline of 75% in its neutral fat concentration to low levels which persisted until death.

By the 21st day, the weight of the tumor was over 20% of that of the tumor–host organism. There were no further marked changes in lipid metabolism apart from some accumulation, apparently from extraneous sources, of free cholesterol in the central core, which by this time was beginning to appear degenerative but did not differ appreciably from the body of the tumor in lipid concentration.

At or near death of the tumor–host organism on the 28th day, with the tumor comprising over 30% of the weight of the organism, there was still no further marked change in lipid concentration in the body of the tumor. The central core appeared definitely necrotic but the only difference from the body of the tumor found on lipid analysis was some decrease in phospholipid concentration.

Thus, throughout its life history, the only marked change in lipid concentration of the tumor component of the tumor–host organism was the early decline in neutral or storage fat values. Up to the point of death, the demands of the tumor component of the tumor–host organism were for maintenance at all costs of some 6 to 8 gm. of phospholipid, 1 to 2 gm. of free cholesterol, 1 to 2 gm. of ester cholesterol, 2 to 3 gm. of total cholesterol, 9 to 11 gm. of total fatty acids, 3 to 6 gm. of neutral fat, and 14 to 17 gm. of total lipid per 100 gm. dry weight of tissue. These may be considered as minimal critical concentrations maintained in spite of an obvious visible progressive loss of large amounts of fat from adipose tissues of the host component.

So much for concentration. In addition, one must consider the accumulation of lipids in the tumor component of the Walker Carcinoma 256–albino rat organism. From the data listed in Tables I, II, and III, an estimate of the lipids accumulated in the tumor may be calculated. For the purpose of making this calculation, it has been assumed that the lipid concentrations of the tumor were those of the body of the tumor as listed in Table III, a reasonably correct assumption except for phospholipid values at the end of 28 days which will be not quite correct. These values were calculated for the end of each week of tumor growth, the results expressed as grams of tumor lipid per kilogram body weight of the tumor–host organism and the figures assembled in Table V.

It will be obvious that after seven days of tumor growth, a negligible proportion of the lipids of the tumor–host organism was contained in the tumor component. Between the 7th and 14th days, the grams of total lipid in the tumor, per kilogram body weight of the dual organism, rapidly increased to

eight and one-half times the former amount. In the next week, the rate of increase in tumor total lipid was two times and in the last week one and one-half times that of the previous week respectively. The increasingly appreciable accumulation of lipids in the tumor component of the tumor–host organism from the 14th to 28th day may be seen to have been due principally to increasing accumulation of phospholipid, free cholesterol, and cholesterol esters. During this latter three-quarters of the growth period of the tumor, the accumulation of neutral fat remained fairly constant at about 1 gm. per kilogram of tumor–host, but the accumulation of phospholipid rose from 1.11 to 3.92 gm., of free cholesterol from 0.19 to 0.61 gm., of ester cholesterol from 0.13 to 0.42 gm., yielding accumulations of total lipid from 2.38 to 6.50 gm. per kilogram of tumor–host in the tumor component. In subsequent studies, it is planned to find how these accumulations of lipids in the tumor component affected the lipid content of the host component and how lipid content of the tumor–host organism varied from that of nontumorous albino rats.

Acknowledgments

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WALLERIAN DEGENERATION IN THE RAT
A CHEMICAL STUDY[1]

By W. A. Mannell

Abstract

The wet weight of the sciatic nerve of the rat increased as the nerve degenerated after nerve section. At the same time the concentration of nucleic acid increased and the concentration of total phospholipid, sphingomyelin (phosphosphingoside), and free cholesterol decreased. Cholesterol ester, absent from intact nerves, appeared during the degeneration. These findings are in agreement with those previously reported for the sciatic nerve of the cat (3, 5), with the exception that all the changes took place more rapidly in the rat than in the cat. It would thus appear that the changes previously described for the cat are general in nature and not merely characteristic of one particular species. These experiments indicate that the rat is a suitable experimental animal for the study of the effect of dietary and other factors on Wallerian degeneration.

Introduction

If a peripheral nerve is cut, that portion of the nerve distal to the point of section undergoes a series of changes known as Wallerian degeneration. Previous papers from this laboratory have described the changes that occur in the concentration of lipids (3), nucleic acids, and other protein-bound phosphorus compounds (5) in the sciatic nerve of the cat during such a degeneration. After a delay of about eight days there is a fall in the concentration of total phospholipid, sphingomyelin, and free cholesterol, a fall that is most rapid during the period 8-32 days after the nerve section. During this time ester cholesterol, which is absent from intact nerves, appears for the first time (3). There is also an increase in the concentration of nucleic acid, which is greatest 16 days after the operation. This increase is statistically significant as early as two days after nerve section, i.e. well in advance of the changes in the concentration of the lipids (5).

Since it was desired to extend these studies to include an investigation of the effect of dietary deficiencies and other factors on nerve degeneration, it became necessary to know whether the rat, which in many respects is a more suitable animal for nutritional and endocrinological experiments, showed the same sequence of changes as the cat. This paper describes changes in the concentration of total phospholipid, sphingomyelin, total, free, and ester cholesterol, and total nucleic acid in the sciatic nerve of the rat after nerve section. The results in every way confirm our earlier work with the cat with the exception that, as was to be anticipated, the successive changes in the rat nerve were considerably speeded up.

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Contribution from the Department of Biochemistry, University of Western Ontario, London, Ont. The work was supported by grants from the National Research Council of Canada and the National Mental Health Grants.

Methods

The right sciatic nerve of each of 62 rats was sectioned high in the thigh. The operation was performed using aseptic precautions under ether anesthesia. The two cut ends were separated as far as possible with a muscle mass. The animals were of the same strain and, with the exception of two, all were females. The figures for the two males fell within the range found for the females and have been included in the results. After 1, 2, 4, 8, 16, and 32 days, groups of animals were sacrificed and the distal degenerating segment of the sciatic nerve was removed for analysis. All of the nerves were examined for evidence of regeneration. In only three animals, all from the 32-day group, was there any sign of a connection between the proximal and distal stumps. Although the figures for these animals fell within the range found for animals showing no evidence of regeneration, they have been excluded from the results. A similar length of left sciatic nerve was taken from each animal to serve as a control. Each nerve was rapidly cleaned of adherent fatty and epineurial connective tissue and weighed on a torsion balance. The nerve was then homogenized in a Potter–Elvehjem type homogenizer in 3 ml. ethanol. The lipids were removed from the homogenate by extracting twice with 5 ml. ethanol, three times with 5 ml. of a 1 : 3 ether–ethanol mixture heated to boiling for three minutes, and once with 5 ml. ether. The acid-soluble compounds and the nucleic acids were then successively removed with cold and hot trichloroacetic acid as described by Schneider (6).

The combined lipid extract was evaporated under reduced pressure in an atmosphere of nitrogen at 60° C. The residue was taken up in successive small quantities of petroleum ether (b.p. 40-60°) and the petroleum ether was evaporated almost to dryness. The phospholipids were precipitated with acetone and a few drops of a saturated solution of magnesium chloride in ethanol. After centrifuging, the clear supernatant fluid was used for the determination of free and total cholesterol by the method of Schoenheimer and Sperry (7). The precipitated phospholipids were dissolved in a 1 : 1 methanol–ether mixture and total phospholipid and monoaminophospholipid were determined as described by Hack (1). Phosphorus was determined by the method of King (4). The factor of 25 was used to convert lipid phosphorus to phospholipid. Ester cholesterol was calculated as the difference between the concentration of total cholesterol and that of free cholesterol. Sphingomyelin (phosphosphingoside) was calculated as the difference between the concentration of total phospholipid and that of monoaminophospholipid. Details of the procedures are given by Johnson, McNabb, and Rossiter (2).

Total nucleic acid was determined in the Schneider (6) trichloroacetic acid extract by the ultraviolet absorption method described by Logan, Mannell, and Rossiter (5).

Because of the great increase in the wet weight of degenerating nerve, the results have all been expressed in terms of the fresh weight of a similar length of the intact nerve of the opposite side. This method of recording results, which has been used in previous paper (3, 5), is equivalent to expressing the

results for the degenerating nerve in terms of the fresh weight of the nerve before it had been sectioned, i.e. at zero time.

Results

Nucleic Acids

Table I shows that the nucleic acid increased in concentration very quickly after nerve section. The maximum value was observed 16 days after the operation, at which time the mean concentration in the degenerating nerves was nearly five times that in the control nerves. By 32 days the concentration of nucleic acid had commenced to fall, but it was still almost four times that of the control nerves. As little as 24 hr. after the operation the concentration of nucleic acid in the sectioned nerves was significantly ($P < 0.01$) greater than that in the control nerves. Fig. 1 shows that for the rat the increase in the concentration of nucleic acid occurred more rapidly than the similar change reported by Logan *et al.* (5) for the cat.

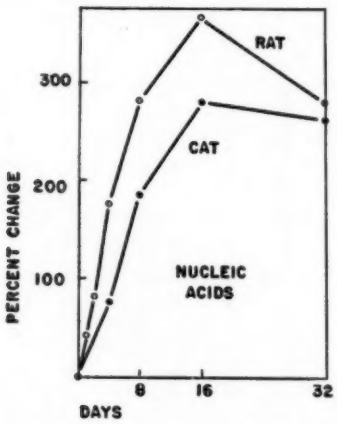

FIG. 1. Mean percentage change in concentration of nucleic acid in the sciatic nerve of cat and rat during Wallerian degeneration. Abscissa, days after nerve section.

Phospholipids

The concentration of total phospholipid did not change during the first two days after nerve section (Table I). After four days the mean concentration in the degenerating nerves was less than that in the control nerves, but the difference was not statistically significant ($P = 0.1 - 0.2$). It was not until eight days that a significant ($P < 0.01$) difference was observed. Fig. 2 shows that the greatest decrease in the concentration of phospholipid was during the period 4-16 days. This was in contrast to previous findings for the cat where the concentration of phospholipid did not change greatly during the first eight days and then fell rapidly during the period 8-32 days (3, 5).

TABLE I

CHANGES IN THE CHEMICAL COMPOSITION OF RAT NERVE AFTER NERVE SECTION. FIGURES REPRESENT MEAN ± S.E. MEAN. THE NUMBER OF ANIMALS IN EACH GROUP IS GIVEN IN BRACKETS

	Days after nerve section						
	0	1	2	4	8	16	32
Total nucleic acid (mgm. P/100 gm.)	13.0 ± 0.23 (60)	18.5 ± 1.24 (10)	23.5 ± 1.75 (8)	35.6 ± 2.50 (9)	49.4 ± 1.88 (9)	60.5 ± 3.24 (13)	49.3 ± 3.79 (7)
Total phospholipid (mgm. /100 mgm.)	8.61 ± 0.12 (59)	8.63 ± 0.33 (9)	8.36 ± 0.60 (8)	8.08 ± 0.36 (9)	6.11 ± 0.21 (8)	3.34 ± 0.20 (13)	2.00 ± 0.16 (7)
Sphingomyelin (mgm. /100 mgm.)	1.46 ± 0.09 (57)	1.88 ± 0.28 (9)	1.45 ± 0.40 (7)	1.43 ± 0.19 (9)	0.69 ± 0.28 (7)	0.47 ± 0.08 (12)	0.26 ± 0.06 (7)
Total cholesterol (mgm. /100 mgm.)	3.86 ± 0.05 (41)	4.04 ± 0.17 (6)	3.81 ± 0.19 (7)	3.59 ± 0.16 (6)	3.47 ± 0.15 (8)	2.48 ± 0.12 (9)	1.30 ± 0.07 (3)
Free cholesterol (mgm. /100 mgm.)	3.80 ± 0.06 (41)	3.97 ± 0.18 (6)	3.69 ± 0.21 (7)	3.31 ± 0.17 (6)	2.66 ± 0.08 (8)	1.42 ± 0.11 (9)	0.75 ± 0.10 (3)
Ester cholesterol (mgm. /100 mgm.)	0.06 ± 0.02 (41)	0.07 ± 0.08 (6)	0.12 ± 0.01 (7)	0.27 ± 0.08 (6)	0.81 ± 0.11 (8)	1.06 ± 0.09 (9)	0.55 ± 0.01 (3)
Wet weight of nerve (mgm.)	24.6 ± 0.6 (62)	25.0 ± 1.0 (10)	29.1 ± 2.2 (10)	30.5 ± 1.6 (9)	37.1 ± 1.6 (10)	37.2 ± 2.3 (13)	37.6 ± 2.9 (8)

Table I shows that the concentration of sphingomyelin followed a course similar to that for total phospholipid, a finding that was previously reported for the cat (3).

Cholesterol

Table I shows that free cholesterol, like phospholipid, did not change greatly in concentration during the first two days, but that by four days the concentration in the sectioned nerves was significantly ($P < 0.01$) less than that in the control nerves. The concentration of free cholesterol then fell rapidly during the period 4-16 days. Fig. 3 shows that this also was different from

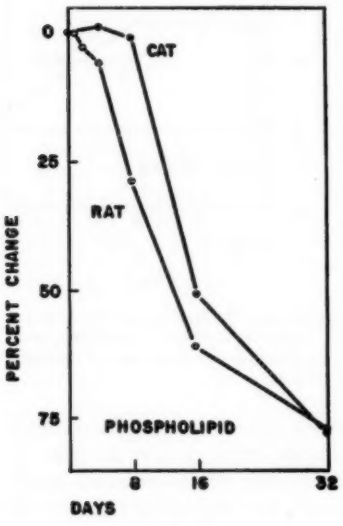

FIG. 2.

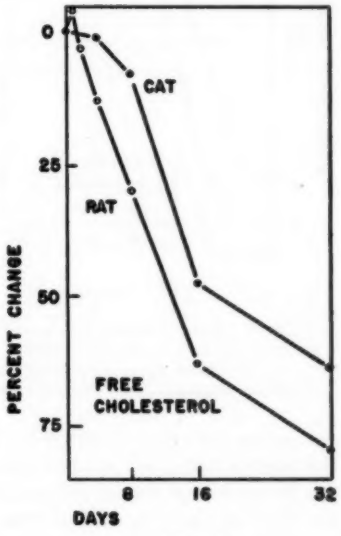

FIG. 3.

FIG. 2. Mean percentage change in concentration of total phospholipid in the sciatic nerve of cat and rat during Wallerian degeneration. Abscissa, days after nerve section.

FIG. 3. Mean percentage change in concentration of free cholesterol in the sciatic nerve of cat and rat during Wallerian degeneration. Abscissa, days after nerve section.

the previous findings for the cat, where the concentration changed but little during the first eight days and then fell rapidly during the period 8-32 days (3). There was no ester cholesterol in the control nerves, but by four days a significant ($P < 0.02$) amount of ester cholesterol had appeared. Fig. 4 shows that, with the exception of the shorter time interval, these findings are in accord with those previously reported for the cat.

Wet Weight

Table I shows that there was no significant change in the wet weight of the degenerating rat sciatic nerve 24 hr. after nerve section, but that by

48 hr. the weight of the degenerating nerve had increased significantly ($P < 0.05$). Fig. 5 shows the percentage change in the wet weight of the sciatic nerve of the rat compared with that of the cat. The figures for the

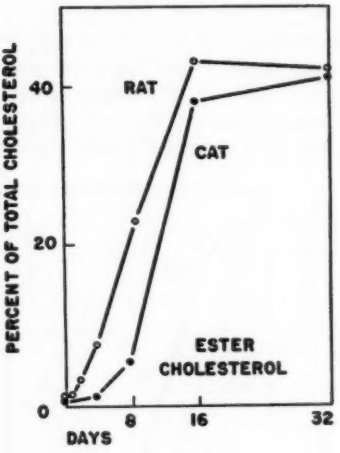

FIG. 4.

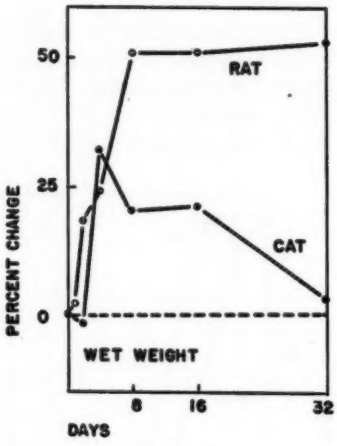

FIG. 5.

FIG. 4. Ester cholesterol (expressed as a percentage of total cholesterol) in the sciatic nerve of cat and rat during Wallerian degeneration. Abscissa, days after nerve section.

FIG. 5. Mean percentage change in wet weight of the sciatic nerve of cat and rat during Wallerian degeneration. Abscissa, days after nerve section.

cat nerves are those reported by Johnson *et al.* (3), to which have been added the figures obtained in the experiments described by Logan *et al.* (5). It can be seen that the initial increase in the wet weight was more rapid in the rat than in the cat. After four days, however, the wet weight of the cat nerves had commenced to fall, whereas the wet weight of the rat nerves continued to increase, reaching a weight some 50% greater than that of the control nerves. The nerves remained at this weight until 32 days after the operation.

Discussion

As far as we are aware, the literature contains no report of a systematic chemical study of Wallerian degeneration in the rat. The results in general confirm those reported previously for the sciatic nerve of the cat (3, 5) and can be similarly interpreted. The increase in wet weight indicates an oedema in the degenerating nerve. The increase in the concentration of nucleic acid represents a cellular proliferation (chiefly of Schwann cells, fibrocytes, and macrophages) and the decrease in phospholipid, sphingomyelin, and cholesterol represents the destruction of the myelin sheath. The difference in the time relations between the rat and the cat was to be expected because of the difference in metabolic rate between the two species.

As previously reported for the cat (5), the changes in the concentration of nucleic acid in degenerating rat nerve (significant after 24 hr.) occurred much earlier than the changes in the concentration of phospholipid (not significant until eight days) or free cholesterol (not significant until four days).

The finding that the changes in the concentration of nucleic acid and lipids in the sciatic nerve of the rat are similar to those previously investigated in greater detail for the sciatic nerve of the cat indicates that the rat is a suitable experimental animal for further projected work on dietary and other factors affecting Wallerian degeneration.

Acknowledgments

Thanks are due to Prof. R. J. Rossiter for his interest in this work. Miss B. Cram and Mr. E. Middleton rendered technical assistance.

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NUCLEODEPOLYMERASE ACTIVITY IN REGENERATING RAT LIVER[1]

By Roger Daoust, Gaston de Lamirande, and Antonio Cantero

Abstract

The desoxyribonucleodepolymerase and ribonucleodepolymerase activity has been estimated in rat liver at various time intervals following partial hepatectomy. When expressed per a constant amount of nitrogen of the tissue, as routinely done, the activity of these enzymes shows significant variations in regenerating rat liver. However, these variations are no longer evident when the enzymatic activity is recalculated "per cell" and it is concluded that they do not reflect actual variations of enzymatic activity in the average cell of the regenerating rat liver. Presumably, the increase in activity of the two nucleodepolymerases, previously observed in liver of rats fed *p*-dimethylaminoazobenzene, should be attributed to the liver injury by the azo dye rather than to the ensuing regeneration with ultimate hepatoma formation.

The feeding of *p*-dimethylaminoazobenzene (DAB) and similar agents to rats produces liver damage followed by regeneration and hepatoma formation. During the initial stage of DAB feeding, the desoxyribonucleodepolymerase (DNAse) of the liver, as well as the ribonucleodepolymerase (RNAse), shows an increase in activity (1, 2). Later, with hepatoma resulting from a more prolonged action of the azo dye, the activity returns to normal (1, 4). Since it is possible that the increased enzymatic activity is associated with liver regeneration, the influence of rapid regeneration on the nucleodepolymerase activity of the liver was investigated following partial hepatectomy.

Methods

Albino rats (175–200 gm.), fed on Purina Fox chow, were partially hepatectomized according to the method of Higgins and Anderson (6). At various intervals following partial hepatectomy, DNAse and RNAse activity of the regenerating liver was determined by methods previously described (4, 5). Controls of enzymatic activity in normal rat liver were carried out with each series of experiments.

The nucleic acid substrates were used in the form of *(a)* sodium salt prepared from desoxyribonucleic acid as supplied by Dougherty Chemicals, N.Y.:— the sodium salt was precipitated out of a slightly alkaline solution with absolute alcohol and washed twice with absolute alcohol and with ether,— and (*b*) sodium salt of ribonucleic acid, as supplied by Schwartz Laboratories, N.Y.

The determinations of DNAse activity are performed on tissue extracts containing 1.23 mgm. of nitrogen per cc. The activity is expressed in terms of percentage decrease of viscosity, after incubation for half an hour at 30° C.,

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of a mixture obtained by adding 2.5 cc. of 1% sodium desoxyribonucleate to 2.5 cc. of the extract. Viscosity measurements were done with Ostwald-Fenske type viscosimeters.

Tissue samples for RNAse activity contained 0.40 mgm. of nitrogen per cc. The activity is expressed in terms of increase in mgm. of acid-soluble phosphorus, after incubation for two and five hours at 37° C., in a mixture containing 1 cc. of 3% sodium ribonucleate, 1 cc. of veronal buffer at pH 7.0, and 1 cc. of the tissue extract.

Experimental Results

The DNAse activity of the regenerating rat liver (Table I) shows a significant decrease at one day after hepatectomy ($P < 0.05$) and a significant increase at 12 days ($P < 0.02$).

TABLE I

ACTIVITY OF DESOXYRIBONUCLEODEPOLYMERASE IN REGENERATING RAT LIVER

(Tissue extracts containing 1.23 mgm. N per cc.)

Days of regeneration	Number of animals	DNAse activity*
0	30	10.4
1	8	8.1
2	6	8.3
3	9	9.9
4	7	12.2
6	9	11.6
8	5	12.5
10	9	12.6
12	6	13.1
24	3	10.4

* *Definition of activity given in text.*

TABLE II

ACTIVITY OF RIBONUCLEODEPOLYMERASE IN REGENERATING RAT LIVER

(Tissue extracts containing 0.40 mgm. N per cc.)

Days of regeneration	Number of animals	RNAse activity*	
		Period of incubation	
		Two hours	Five hours
0	10	.10	.28
1	7	.10	.31
2	10	.11	.34
4	10	.11	.46
6	9	.12	.38
8	6	.10	.26
12	6	.11	.31

* *Definition of activity given in text.*

There is an apparent increase in RNAse activity at various intervals, following incubation of the mixture for two hours, which is not statistically significant (Table II). However, following incubation for five hours, a statistically significant increase in RNAse activity is found at four and six days after hepatectomy ($P < 0.001$ and < 0.02 respectively).

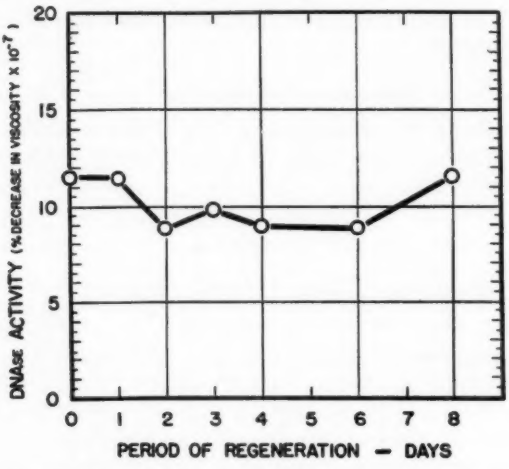

FIG. 1. Activity of desoxyribonucleodepolymerase per cell of regenerating rat liver—computed using the data of Price and Laird (8) for the number of nuclei per gram of fresh liver.

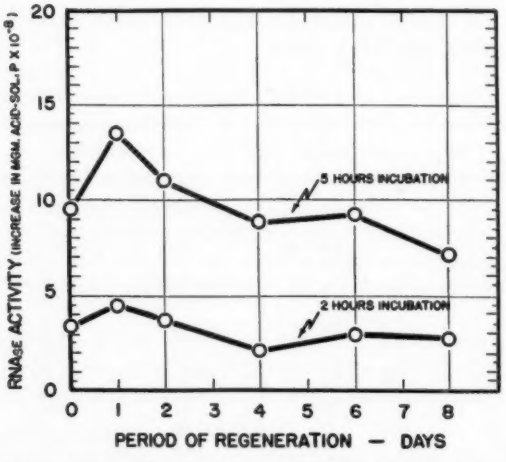

FIG. 2. Activity of ribonucleodepolymerase per cell of regenerating rat liver—computed using the data of Price and Laird (8) for the number of nuclei per gram of fresh liver.

Discussion

The usual methods of computing the enzymatic activity of a tissue, on the basis of nitrogen content of the tissue, wet weight, dry weight, etc., raise the question concerning the extent to which these methods express the variations which take place in the average cell of a tissue. In recent years, there has been a tendency to express the concentration of chemical compounds in various tissues as the content "per cell", and it would seem that enzymatic activities could also be more adequately expressed on this basis. An attempt was therefore made to calculate the nucleodepolymerase activity "per cell'. This calculation is based on results reported by Price and Laird (8) for the number of nuclei per gram of fresh liver at various intervals after partial hepatectomy. The enzymatic activities per cell are presented in Figs. 1 and 2. Since the values for enzymatic activities and values for the number of nuclei per gram of fresh liver have been obtained in two different laboratories, no statistical analysis was possible. However, the general trend of the results is quite different as it is when the nucleodepolymerase activity is expressed per a constant amount of nitrogen. The statistically significant variations as noted in Tables I and II are no longer evident for enzymatic activity when computed per cell. This may probably be explained by the wide variations in the amount of nitrogen per cell recently observed in regenerating rat liver (8). Thus, the apparent variations of nucleodepolymerase activity observed in Tables I and II seem to be mainly attributable to variations in the amount of nitrogen per cell and therefore do not reflect actual changes of enzymatic activity in the average cell of regenerating rat liver.

The nucleodepolymerase activity in the liver of rats fed *p*-dimethylamino-azobenzene, computed on nitrogen basis, has been reported previously (1, 2.) The increase in DNAse and RNAse activity, at the 90 days' interval, was estimated to be of the order of 75 and 150% respectively. The values for the number of nuclei per gram of fresh liver at various intervals during feeding DAB have been recently estimated in this laboratory (3) and on these results the nucleodepolymerase activity per cell has been computed for liver of rats fed DAB in a manner similar to that for regenerating rat liver. The curves obtained were the same, whether the activity was computed per cell or per a constant amount of nitrogen. In recalculating the values, the increase in enzymatic activity at the 90 days' interval was found to be of the order of 60% for DNAse and 135% for RNAse. This increase in activity covers approximately a period extending from the 30 days' to the 150 days' intervals (with a maximum activity at 90 days) which, when correlated with histological findings (7), suggests that the increased activity could be related to a degenerative process occurring in the liver.

An increase in nucleodepolymerase activity similar to the one observed in the early period of DAB feeding is not observed in the process of regeneration following partial hepatectomy. It thus appears that the increase in enzymatic

activity during the process of carcinogenesis is probably a consequence of liver injury due to *p*-dimethylaminoazobenzene rather than that of ensuing regeneration.

Acknowledgments

The authors wish to thank Misses Louise Bourbeau and Paule Panet-Raymond for valuable technical assistance and Mr. Yvon Grandchamps for statistical analysis of the results.

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RADIO-FREQUENCY REWARMING IN RESUSCITATION FROM SEVERE HYPOTHERMIA[1]

By W. G. Bigelow,[2] J. A. Hopps,[3] and J. C. Callaghan[2]

Abstract

Twenty-seven dogs and monkeys were restored to normal body temperature from near-lethal limits of cold, using a radio-frequency rewarming technique. Induction cable applicators were chosen for their facility of arrangement and comparative safety. There was no evidence of optimum frequency among the three radio frequencies used. However, the rate of rewarming was dependent upon the spacing of coils from the body, with most satisfactory rewarming resulting from the use of ½ in. thick rubber pads. Dogs were rewarmed at an average rate of 11.1° C. per hour, using the ½ in. spacing and a frequency of 13.56 megacycles per second.

Introduction

The rapid rewarming of animals whose body temperatures have been lowered to near-lethal limits has been found the most effective method of resuscitation in previous studies of general hypothermia (1, 2, 3). Total immersion in water at 40° C. had become our standard method of rewarming. However, the rewarming of superficial tissues before the deeper structures, which is a result of this technique, has always been suspected as a cause of the fetal cardiovascular collapse sometimes encountered during resuscitation.

The use of diathermy to produce elevation of body temperature above normal suggested the application of a radio-frequency technique of resuscitation from severe hypothermia. In particular, the deep tissue heating characteristic of radio-frequency therapy offers a method of avoiding large temperature gradients between the peripheral and deeper tissues and perhaps might eliminate cases of apparent peripheral vascular collapse. A review of the literature showed no reports of the use of radio-frequency rewarming for severe hypothermia.

The increased military significance of the arctic regions has directed our investigations to a study of survival from exposure to extreme temperatures of air and water. Consideration of a possible military application necessitated a resuscitation technique applicable in the field or on board ship. A complication of exposure to severe cold in war-time will be the presence of wounds and frost-bite of the extremities. It was felt that a cabinet or blanket enclosed coil would be a more satisfactory method of handling resuscitation, in such cases, than immersion rewarming.

The purpose of this investigation has been to develop a safe radio-frequency resuscitation procedure requiring only a small portable radio-frequency generator and power supply with a simple technique of application. Our

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Contribution from the Department of Surgery, University of Toronto, Toronto, Ont.

[2] *Department of Surgery, University of Toronto.*
[3] *Radio and Electrical Engineering Division, National Research Laboratories, Ottawa, Canada.*

early investigations of diathermy rewarming indicated the need of technical assistance. This was obtained from the National Research Council of Canada.

Dogs and monkeys were cooled to around 20° C., which borders on the lethal limit of cooling for these animals. The cooling was done in three ways to simulate a variety of cold conditions.

In preliminary investigations of methods of rewarming, 12 cm. microwave diathermy was first applied. Production of intense local heating resulted, and the technique was abandoned in favor of shortwave diathermy. Experimental tests indicated that conventional capacitive (spaced-plate) heating was unsatisfactory because of the danger of contact burns, and the difficulty of obtaining even heat distribution. Induction cable technique was adopted because of the facility of application and reduced danger of high-frequency burns to both operator and subject.

A Liebel-Flarsheim Model SW-550 diathermy generator was used as a radio-frequency power source. The oscillator circuit was modified to permit variation of output frequency and power. With the induction cable technique used in our experiments, the frequency remained stable throughout the tests.

Methods

(*a*) *Cooling*

Twenty-two unselected mongrel dogs of medium size (6–14 kgm.) and five immature Rhesus monkeys (macagna Lata) were cooled to rectal temperatures of 18°–21° C. and all rewarmed to normal body temperature. Three methods of cooling were used to lower the rectal temperatures of the animals to the required level. Fifteen dogs were cooled in specially constructed blankets* with circulating refrigerant as described in previous studies (1, 2, 3). These animals were close-clipped prior to cooling and given enough pentothal and curare to just control the shivering and permit of satisfactory cooling rates. The dogs were intubated and breathed oxygen during the procedure. Since in dogs respirations cease when the rectal temperature falls below 22°–23° C., positive pressure oxygen was delivered below this temperature. Periodic electric stimulation of the exposed phrenic nerve (9) was used experimentally on four dogs to control respiration during the cooling cycle.

In order to simulate exposure conditions more closely, five dogs and five monkeys were cooled by immersion in ice-cold water (0° C.–10° C.) and two dogs were cooled by exposure to extremely cold air (−22° C.). In these last two methods of cooling, a very small amount of intravenously administered pentothal acted as a temporary sedative until the narcosis of hypothermia occurred, and shivering control was not necessary to permit rapid lowering of body temperature.

During the cooling process, body temperatures were recorded using a resistance thermometer inserted well into the rectum (10–12 cm.) and connected to

* *Thermo-rite Co., Ltd., Buffalo, N.Y., U.S.A.*

a Bristol recorder. During the rewarming sequence of the experiments a mercurial thermometer was inserted the same distance into the rectum. Previous studies (3), confirmed in this investigation, showed that the temperatures in the rectum and in the right auricle do not differ by more than 1° C., from which it was concluded that rectal temperature readings would be satisfactory. The error in thermometer reading introduced by its proximity to the radio-frequency field was always less than 1° C., and in most cases, was negligible.

Continuous observation of heart beats during both the cooling and rewarming phases of the experiment was made possible by use of a cathode ray electrocardiograph* with subcutaneous german silver needle electrodes. A low-pass radio-frequency filter on each lead permitted continuous observation with diathermy in operation. A glass cannula in the femoral artery, attached to a mercury manometer, provided arterial blood pressure measurements. Venous pressures were measured using a hard rubber catheter inserted into the right atrium through the external jugular vein and attached to a heparin–saline manometer.

When the rectal temperatures of the animals reached one centigrade degree above that desired, the animals were removed from the cooling system, dried carefully with towels to avoid subsequent danger of radio-frequency burns, and then allowed to stabilize their rectal temperatures for 30–60 min. before the commencement of rewarming.

(*b*) *Rewarming*

In preliminary tests, four dogs were rewarmed inductively at a frequency of 27.12 megacycles per second and with one or two thicknesses of huck towelling for insulation. In two subsequent rewarmings, spacing was changed to 2-in. thicknesses of sponge rubber. In a further series using the 2-in. spacing, three dogs were rewarmed at a frequency of 14.7 megacycles per second and six at 13.56 megacycles per second. In the procedure finally adopted a frequency of 13.56 megacycles per second was used, with ½-in. rubber pads for spacing. Seven dogs and five monkeys were rewarmed by this method.

Each animal was rewarmed in a diathermy cabinet constructed along the lines of a conventional fever cabinet. The animal was laid on his side. Matching induction coils were used, one placed under the animal and the other over it, each insulated from the body by rubber pads. Ports were provided in the sides of the cabinet for the induction cable and electrocardiograph leads.

The cable structure consisted of two flat coils. With medium-sized dogs (6 to 14 kgm.) single turn coils of 3 to 5 in. widths and of 14 to 18 in. lengths were employed. In rewarming the small monkeys, one and one-half turn coils were used because difficulty was encountered in properly loading small single turn coils. The maximum radio-frequency current was limited to approximately seven amperes.

* *Smith and Stone Co., Georgetown, Ont.*

In three of the dogs, copper constantan thermocouples, were embedded in subcutaneous tissue, muscle, pleural space, right atrium, and rectum, and temperatures were read at intervals using a Brown recorder. The radio-frequency power was turned off during thermocouple readings to eliminate errors induced by the electromagnetic field.

Following the experiment, animals were examined daily for evidence of burns and their behavior was noted. After the first week, this examination was carried out two to three times a week for 4 to 12 weeks. All the monkeys were submitted to behavior tests before and after the experiments.

Results

General

Immediately before rewarming, all 27 animals presented a similar physiological picture. The animals were deeply unconscious without the aid of further anesthetic at the lowered temperature. All but eight had ceased to breathe spontaneously and required positive pressure or electrophrenic respiratory assistance. In each case, the heart had slowed markedly, rates varying between 2 and 60 per minute, the average value being 30 per minute. Mean systolic arterial blood pressure fell to around 50 mm. of mercury. All voluntary movement was absent and no reflexes could be elicited. The oxygen saturation was between 90 and 100% and the tongues appeared bright pink in color.

Rewarming

The resuscitation of these animals with the diathermy technique described proved very satisfactory. In preliminary trials involving local burns and some

TABLE I

RELATIONSHIP OF COIL SPACING TO REWARMING RATE

Series	Number of animals	Frequency (Mc./sec.)	Coil distance from body	Average rewarming rate (C.°/hr.)	Incidence burn
1	4 dogs	27.12	Single towel	10.5 (8.9 to 12.7)	4
2	2 dogs	27.12	2 in. Dunlopillo	7.6 (6.0 to 9.3)	0
3	3 dogs	14.7	2 in. Dunlopillo	7.2 (4.9 to 9.0)	0
4	6 dogs	13.56	2 in. Dunlopillo	7.1 (4.6 to 9.4)	0
5	7 dogs	13.56	0.5 in. rubber net	11.1 (7.0 to 21.8*)	0
6	5 monkeys	13.56	0.5 in. rubber net	17.9 (9.6 to 23.2)	0

* *This rate was for one of the two air-cooled dogs. The other rewarmed at 13.9° C. per hour.*

deaths the two chief variables studied were spacing of the coils and variations in frequency. The distance of the coil from the animal proved important both regarding the rate of rewarming and the incidence of burns. Varying the frequency did not appear to influence the rewarming rates significantly. Series 1 to 4 in Table I demonstrate this.

As a result of this, a standard technique was established. The lower frequency of 13.56 megacycles per second was chosen to reduce the current leakage between coils. A series of seven dogs and five monkeys were rewarmed at this frequency and with ½ in. spacing. The results with no deaths are shown in Table I, Series 5 and 6.

The rates of rewarming are illustrated graphically by two cases shown in Fig. 1. The lines are almost linear to a body temperature of 28° C., then the rise is somewhat more rapid. These rewarming rates were similar to those obtained using immersion in water at 40° C.

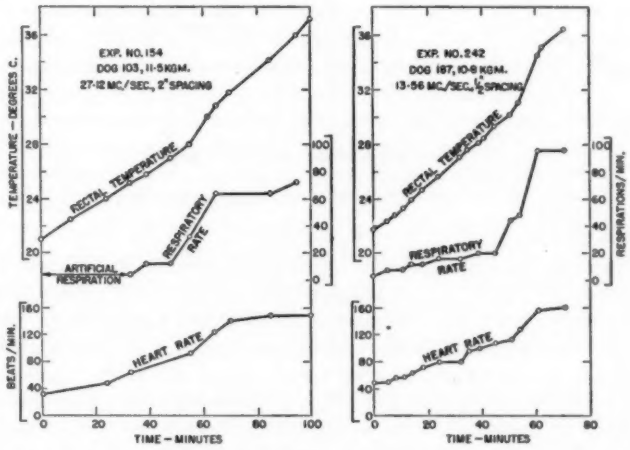

FIG. 1. Relationship of rectal temperature, respiratory rate, and heart rate during radio-frequency rewarming. Fig. 1, *a* at left. Fig. 1, *b* at right.

The animals cooled by blankets and by immersion in cold water rewarmed at the same rate. The air-cooled dogs rewarmed at a faster rate, perhaps because their coats were completely dry.

Relative Rates of Tissue Rewarming

Using embedded copper–constantan thermocouples in the right atrium, pleural space, muscle, subcutaneous tissue, and rectum (see Methods), we were interested to observe that the temperatures conformed rather closely through rewarming. The only exception was found in the subcutaneous tissue which showed a rapid initial rise decreasing in rate to a final temperature one or two degrees above the final temperature of the deeper tissues (See Fig. 2).

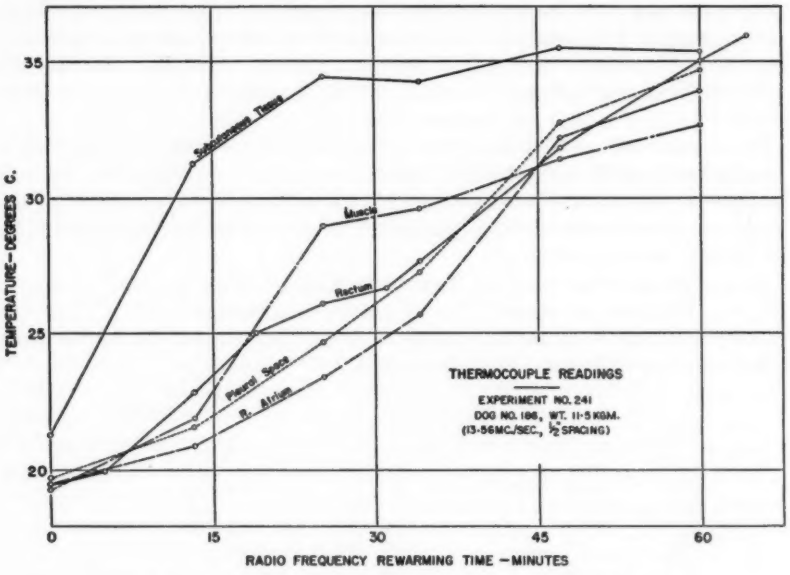

FIG. 2. Relative radio-frequency rewarming rates within the body.

Complications

Local burning was a problem only if insufficient insulation was used. All the dogs of Series 1 (Table I), with a towel as insulation, suffered severe local burns. One-half inch rubber insulation proved to be a safe minimum of protection.

Since the concentration of eddy currents is greatest in areas of high electrical conductivity, it is necessary to keep metal objects out of the electromagnetic field. In two rewarming tests with dogs, we observed local subcutaneous burns at the point of insertion of the needle electrodes for the electrocardiograph, where the electrodes were in the upper legs, within the field of the coils. Relocation of the electrodes in the lower legs eliminated heating of the metal and obviated burns.

Reasonable care was taken to keep the coils from close proximity to the mercury rectal thermometer. Tests indicated that the error introduced by the electromagnetic field was at no time greater than 1° C., and in most cases, was negligible.

When electrophrenic stimulation was used, heating of the silver foil electrode around the phrenic nerve in the neck was slight. It was not necessary to ground the electrode during rewarming.

Deaths

When unselected unprepared mongrel dogs are used as the experimental animal, cooling to 21° C. results in a mortality rate of 10 to 15%. In monkeys cooling is a very safe procedure. All animals studied were returned

to normal body temperature. Two dogs developed ventricular fibrillation when they were hypothermic and would ordinarily have represented mortalities. Their hearts were quickly defibrillated using disk electrodes and a strong countershock current (1, 7) which produced cardiac standstill. The electrical artificial pacemaker (4) was then applied producing good expulsive beats, and the rewarming was continued. In each case, as the animal's temperature approached normal, the heart took over its own rhythm and normal body temperature was restored.

Cardiorespiratory Changes

As the rectal temperature rose, return of heart action, respiration, and cerebral function occurred. By the time 25° C. was reached the heart rate varied from 48 to 100, averaging 70 beats per minute. At 30° C. the rates varied from 80 to 140 beats per minute and at 35° C., from 84 to 160.

Respirations had returned to 70% of the animals at 25° C. rectal temperature, at an average rate of 18 per minute. At 30° C., 85% were breathing spontaneously at rates averaging 33 per minute. At 35° C. all animals were breathing spontaneously at an average rate of 46 per minute. No evidence of cyanosis was seen in any of the dogs being rewarmed (Fig. 1).

Venous pressures varied between +9.0 cm. and −4.5 cm. of saline at the start of rewarming and between 0.8 and 0 at normal body temperature. In five cases observed closely during the rewarming process, the venous pressure fell an average of 3.9 cm. saline.

In three cases in which blood pressure was observed during the rewarming, the systolic pressure rose satisfactorily.

In nine of the dogs in which shivering was noted, the reappearance varied from 21.4 to 29° C., averaging 26.0° C. In seven of the cases, the first struggling movements were recorded at temperatures between 31° C. and 37° C., average value 34.9° C.

Behavior of the dogs and monkeys was observed before and after rewarming with no apparent change in cage behavior. The monkeys were unchanged in their ability to elude captors and in the performance of rake and string tests (5).

Discussion

The radio-frequency technique described would appear to be a satisfactory method of resuscitation in animals. We did not encounter any vascular collapse in the final series of animals, which would suggest that the coincident heating of deep and superficial tissues may eliminate this danger.

The reasonable conformity of temperatures measured with thermocouples in five locations in an animal substantiates the recognized deep heating characteristics of induction therapy. Insufficient investigation was made to attempt any analysis of relative rewarming rates throughout the body. The initial high rewarming rate shown for subcutaneous tissues may have been influenced to some extent by high local field intensities in the vicinity of the metal thermocouples. The thermocouple readings were not affected by the

magnetic field since the radio-frequency power was turned off and sufficient time allowed to obtain stable readings. Perhaps this higher subcutaneous rewarming rate is more an index of the type of tissue than its peripheral location. We have not achieved complete uniformity, but the radio-frequency technique eliminates the marked temperature gradients produced by warm water. Early warming of muscle masses in the limbs with dilation of their vascular bed, which probably occurs in water rewarming, is thus avoided before heart action has returned to accommodate it.

In so far as general body warming is concerned, within the normal induction cable therapy range selection of frequency is not critical.* Our final selection of 13.56 megacycles per second was based solely on reduced capacity leakage between coil turns at the lower frequency. Other investigators have studied "selective" heating (6) of various tissues and locations in the body and their findings show no conclusive evidence of selective heating of one tissue at any particular radio frequency. In general, inductive heating will favor the vascular tissues because of their greater conductivity.

Heat generation varies directly with conductivity, but it also varies with the *square* of the field strength, making uniform distribution of the magnetic field of critical importance. Osborne and Holmquest (8) show that the intensity of the magnetic field within a solenoid coil is reasonably uniform from the axis out to a point about 0.8 of the radius of the coil. Beyond that point it rises sharply to a maximum on the inner edge of the coil.

The severe burns encountered in early rewarmings were due to insufficient coil spacing. Increasing the spacing to two inches from the body removed the subcutaneous tissue area from the intense field. A very even distribution of field intensity resulted, but at a sacrifice of energy absorption, owing to reduced coupling. The final use of one-half inch spacing effected improved coupling without excessive field concentration near the surface of the body. In tests with the one-half inch spacing all animals were healthy survivals.

We were interested to note that the two dogs which were cooled by cold air exposure rewarmed at a much higher rate than the dogs cooled by cold water or refrigeration blanket. The results of only two tests cannot be considered conclusive, but might be explained by the condition of the animals' coats before rewarming. With water-cooled dogs, and to a lesser extent with blanket-cooled dogs, some moisture remained on the surface of the body after drying with towels, presenting a conductive path for current leakage. The dry surface of the air-cooled dogs presented a better condition for the rewarming technique.

The results of our experiments indicate the possibility of a practical resuscitation technique for humans in a hypothermic state from sea immersion or cold

* *Frequencies employed with induction cable diathermy usually fall within the range of 10 to 30 megacycles per second (30 to 10 meters wave length). Power absorption is proportional to the product of the square of the induction cable current and a function of frequency, so that selection of a lower frequency involves excessive current and heating of the coil assembly. On the other hand, as the frequency is increased it approaches the natural frequency of the coil, and the cable-load coupling becomes progressively less inductive and more capacitive owing to leakage currents between coil turns. The leakage path is often through subcutaneous tissue and produces undesirable local heat.*

PLATE I

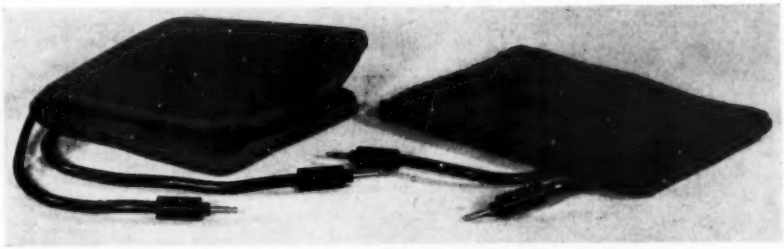

FIG. 3. Blanket rewarming coils (National Research Council of Canada).

air environment. A sleeping bag with enclosed induction coils would facilitate the procedure of rewarming, with the operator having only to apply power from a portable oscillator unit. Two blanket pads have been constructed for use in a preliminary approach to this objective (Fig. 3). The procedure appears to be perfectly safe and dogs as large as 18 kgm. have been rewarmed. Personnel of the team have been rendered hyperthermic by this technique with no sign of superficial burn or undue side effects.

Attention should be drawn to the electrical artificial pacemaker, electrophrenic respirator, and defibrillator which in the future may prove to be valuable accessories in the treatment of severe exposure to cold.

Summary

1. A radio-frequency induction cable technique has been developed which has successfully rewarmed dogs and monkeys from near-lethal hypothermia of 21° C. to normal body temperature.

2. The short wave therapy range was found most effective for heating the deep tissues of large animals.

3. Spacing of coils from body was the most critical rewarming factor. Using one-half inch spacing, adequate heat generation was obtained without burning the subcutaneous tissue.

4. A frequency of 13.56 megacycles and an insulation of one-half inch was finally selected as the safest and most effective combination and became a standard technique.

5. A final series of animals was resuscitated by this technique. They all survived with no burns, no signs of vascular collapse, and no post-rewarming change in behavior or intelligence.

Acknowledgments

This work was financed by a grant from the Defence Research Board of Canada. Technical assistance and electrical equipment were provided by the Division of Radio and Electrical Engineering, National Research Laboratories Ottawa, Canada. Cold chamber facilities and thermocouple equipment were made available through the co-operation of the Institute of Aviation Medicine, Toronto, Ont.

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PREPARATION OF A STABLE NONINFECTIVE SOLUBLE INFLUENZA A ANTIGEN

II. USE OF VARIOUS REAGENTS FOR NEUTRALIZATION OF FORMALDEHYDE-TREATED ANTIGENS[1]

By John R. Polley

Abstract

When soluble influenza antigen is treated with formaldehyde to render it noninfective, the excess formaldehyde present causes a large loss of antigenicity on lyophilization. It has been shown previously that neutralization of the formaldehyde with ammonium hydroxide would overcome this difficulty. In the present study, a wide range of chemical compounds has been investigated to seek other reagents effective in neutralizing the formaldehyde present in treated soluble influenza antigen and which would possibly have advantages over ammonium hydroxide. It has been found that urea, dibasic ammonium phosphate, and sodium bisulphite are as effective as ammonium hydroxide, and possess the added advantage of not requiring any pH adjustment in the process.

Formaldehyde is widely used to destroy the infectivity of numerous rickettsial, viral, and soluble antigens used for diagnostic serological tests. On storage, however, the treated soluble antigens tend to become anticomplementary. Attempts have been made to lyophilize the formaldehyde-treated products but large losses of activity occurred (3, 7), probably due to the denaturing action of the excess formaldehyde. Efforts were then made to neutralize the formaldehyde prior to lyophilization. Sabin (4) found that the formaldehyde-treated vaccines of St. Louis and Japanese encephalitis could be lyophilized if the excess formaldehyde was first neutralized with sodium bisulphite. Ammonium hydroxide was also tried as a neutralizing agent but was found to be less successful. Sodium bisulphite was used later by Smadel *et al.* (6) for the large scale production of Japanese encephalitis vaccine. Dibasic ammonium phosphate was used to neutralize the formaldehyde in the preparation of inactivated Lansing poliomyelitis virus for animal immunization experiments (5).

Recently a method, involving the neutralization of the formaldehyde with ammonium hydroxide prior to lyophilization, was published for the preparation of stable noninfective soluble influenza A antigen (3). In the present study, it was proposed to investigate the use of various compounds for this neutralization process in soluble antigen in order to seek reagents having advantages over ammonium hydroxide. The lyophilized antigens so prepared were tested in the complement–fixation test for specificity and anticomplementary effects. The antigens were also tested for infectivity and storage stability.

Formaldehyde undergoes chemical reaction which may be classified into three types: (1) addition or condensation reactions which lead to the formation of methylene derivatives, (2) oxidation–reduction reactions in which the

[1] *Manuscript received February 25, 1952.*

Contribution from the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

formaldehyde acts as a reducing agent and is itself oxidized to formic acid, and (3) polymerization reactions resulting in the formation of polymethylene derivatives. Many compounds containing hydrogen atoms in the α position with respect to a carbonyl, nitro, or cyano group undergo addition reactions with formaldehyde, thereby forming methylol groups, $-CH_2OH$, in the position formerly held by the active hydrogen. Since the polymerization and oxidation–reduction reactions in general involve reagents and reaction conditions incompatible with the preparation of antigens or vaccines, consideration was given primarily to compounds such as acetaldehyde, nitromethane, urea, sodium bisulphite, ammonia, and ammonium salts, etc. which are capable of addition or condensation with formaldehyde.

Materials and Methods

The soluble antigen used was prepared from the chorioallantoic membranes of 12-day-old chick embryos which had been infected with the PR8 strain of influenza A virus by the allantoic route two days previously. To the pooled membranes was added 1.5 ml. of saline per membrane and the mixture was blended for two minutes with a Waring blendor. This extract was centrifuged for 10 min. at 2000 r.p.m. and then the supernatant suspension centrifuged again for one hour at 15,000 r.p.m. The supernatant liquid was removed and used as the soluble antigen.

Reagent grade chemicals were used. When the reagent to be used was a solid, it was dissolved in distilled water so that the addition of 0.1 ml. of its solution to 10 ml. of antigen containing 0.1% formaldehyde would be sufficient to provide the theoretically required amount of the reagent to react with all the formaldehyde. Liquid reagents were diluted in distilled water to similar concentrations. Where the solubility of the reagent was too low to reach these concentrations, as with nitromethane and ammonium oxalate, they were added directly to the formaldehyde-treated antigen. Calculations were based on the following reactions with formaldehyde:

$$CH_3NO_2 + CH_2O \xrightarrow[K_2CO_3]{} (HOCH_2)_3CNO_2$$

$$H_2NCONH_2 + 2\ CH_2O \longrightarrow HOCH_2NHCONHCH_2OH$$

$$NaHSO_3 + CH_2O \longrightarrow HOCH_2SO_3Na \quad (2)$$

$$4NH_3 + 6\ CH_2O \longrightarrow (CH_2)_6N_4 + 6\ H_2O$$

$$4NH_4Cl + 6\ CH_2O \longrightarrow (CH_2)_6N_4 + 6\ H_2O + 4\ HCl$$

For example, the following concentrations were used: urea (20%), dibasic ammonium phosphate (30%), ammonium hydroxide (5%), and sodium bisulphite (35%).

A common pool of soluble antigen was treated with formaldehyde to render it noninfective by the procedure previously found to be effective for this

purpose, i.e., 0.1% formaldehyde at 37° C. for two hours (3). The formaldehyde was added in the form of a 10% solution. Ten milliliter aliquots of the treated antigen were then placed in test tubes in a water bath at 25° C. for 30 min., at which time the reagents to be tested were added. At the end of a further 30 min. the pH of all samples was adjusted to 7.2 – 7.4. Aliquots of each sample were lyophilized and reconstituted to their original volume with distilled water when tested in the complement–fixation test.

TABLE I

ABILITY OF VARIOUS REAGENTS TO NEUTRALIZE THE FORMALDEHYDE IN SOLUBLE ANTIGEN FOR LYOPHILIZATION

Reagent used	CF titer after lyophilization	Anticomplementary titer
Antigen (untreated), control	32	0
Antigen (treated, not neutralized)	< 2	0
Ammonium hydroxide	32	0
Ammonium chloride	16	2
Ammonium sulphate	16	2
Ammonium oxalate	16	0
Ammonium phosphate, dibasic	32	0
Methylamine	32	4
Nitromethane	2	0
Sodium bisulphite	32	0
Urea	32	2
Acetaldehyde	< 2	0

In Table I are shown the results of an experiment in which the theoretical amount of the various reagents required to react with all the formaldehyde was added.

It can be seen from these results that all the reagents tested, with the exceptions of nitromethane and acetaldehyde, were quite effective in neutralizing the excess formaldehyde prior to lyophilization. The best reagents were ammonium hydroxide, dibasic ammonium phosphate, sodium bisulphite, and urea. This experiment was repeated on different lots of PR8 soluble antigen. On each occasion a slight anticomplementary activity of the antigen was noted after treatment with the more acidic ammonium salts. This effect was not observed with dibasic ammonium phosphate or sodium bisulphite tested under similar conditions.

The experiment was repeated as before but using two to four times the theoretically required amount of the various reagents in an attempt to increase the efficiency of the ammonium salts and also to observe any new anticomplementary effects or antigen destruction caused by an excess of the reagent. A few of the reagents such as sodium bisulphite and urea were also added as solids. In addition, the pH of aliquots of each solution involving the use of an ammonium salt was adjusted to 7.5 at the start of the neutralization process. On the other hand, no pH adjustment was made with the antigens neutralized with sodium bisulphite, urea, or dibasic ammonium phosphate.

As controls, a common pool of normal membrane antigen was treated similarly with formaldehyde and samples were treated with the neutralizing reagents under test. None gave significant nonspecific fixation or anticomplementary effects except methylamine and urea and these only to a titer of 1 : 4. In Table II are shown the results when twice the theoretically required amount of the reagents was added.

TABLE II

ABILITY OF VARIOUS REAGENTS TO NEUTRALIZE THE FORMALDEHYDE IN SOLUBLE ANTIGEN WHEN ADDED IN EXCESS

Reagent used	CF titer after lyophilization	Anticomplementary titer
Antigen (untreated), control	64	0
Antigen (treated, not neutralized)	< 2	0
Ammonium hydroxide	64	0
Ammonium chloride	32	2
Ammonium chloride, pH 7.5	32	0
Ammonium sulphate	64	2
Ammonium sulphate, pH 7.5	32	0
Ammonium phosphate, dibasic	64	0
Methylamine	64	4
Acetaldehyde	2	0
Sodium bisulphite (solution)	64	0
Sodium bisulphite (solid)	64	0
Urea (solution)	64	0

From Table II it can be seen that again the ammonim salts of strong acids gave slight anticomplementary effects and were slightly less efficient at preserving the titer of the antigen than ammonium hydroxide, dibasic ammonium phosphate, sodium bisulphite, and urea. Increasing the pH of the solution during the neutralization process eliminated the anticomplementary effect but did not increase the efficiency of the salts in preserving the titer of the antigen as well as ammonium hydroxide. In effect the pH adjustment promotes the reaction with formaldehyde by neutralizing the liberated hydrogen ions (1). This pH adjustment was not necessary with dibasic ammonium phosphate since it yields a mildly alkaline solution. An increasing concentration of methylamine caused the antigen to become increasingly anticomplementary. Sodium bisulphite gave the best results when added in the form of a solution in amounts up to twice that theoretically required but it sometimes gave anticomplementary effects when added in excess as the solid reagent. Urea was an efficient reagent in both the solid and solution form.

In the last experiment, the lyophilized antigens which had been neutralized with ammonium hydroxide, dibasic ammonium phosphate, urea, or sodium bisulphite were reconstituted to their original volume with distilled water and then tested for infectivity. Infectivity tests of control and treated samples were conducted by direct inoculation of undiluted and 10^{-2} diluted solutions

into the amniotic sac of 11 – 12-day-old chick embryos and by a subsequent incubation for 72 hr. Samples, which after two successive amniotic passages yielded amniotic and allantoic fluids free of specific hemagglutinins, were considered to be noninfective. After lyophilization, the ID_{50} of the untreated antigen was found to be approximately $10^{-6.7}$ per ml. by allantoic titration. The treated antigens all proved to be noninfective and to have lost no antigenicity in the process. They were also tested in the complement–fixation test with normal hamster serum instead of specific influenza A serum and gave no nonspecific fixation.

Summary

When formaldehyde-treated antigens are lyophilized directly for stable storage, the excess formaldehyde present causes a large loss of antigenicity. It has been shown previously that the addition of ammonium hydroxide to the treated soluble antigen prior to lyophilization would overcome this difficulty. In the present study it has been shown that numerous ammonium salts, methylamine, sodium bisulphite, and urea, as well as ammonium hydroxide, are effective to varying degrees. Of the compounds tested, only nitromethane and acetaldehyde were of no value. The acidic ammonium salts, such as ammonium chloride, nitrate, and sulphate, are partially effective but the resulting lyophilized antigens are slightly anticomplementary. Increasing the pH of the antigen solution to 7 – 7.5 during the process of neutralization with these salts eliminated the anticomplementary activity of the antigens after freeze–drying. However, this adjustment in pH did not prevent a decrease in antigenicity when using these ammonium salts instead of ammonium hydroxide. Of the ammonium salts tested only dibasic ammonium phosphate was as satisfactory as ammonium hydroxide. It possessed the advantage over ammonium hydroxide that no final pH adjustment was necessary. Methylamine was partially effective but it produced anticomplementary effects which increased rapidly when it was present in excess. Sodium bisulphite and urea were as efficient as ammonium hydroxide in preserving the antigenicity of treated soluble antigen after lyophilization and neither required any pH adjustment in the process.

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SOME ABSORPTION AND FLUORESCENT PROPERTIES OF THE α-ESTRADIOL SULPHURIC ACID COMPLEX[1]

By J. H. Linford

Abstract

Three processes take place when α-estradiol is dissolved in concentrated sulphuric acid: (1) a reaction involving the C_3—OH group, characterized by the development of an absorption maximum at 220$\tilde{\upsilon}$, (2) a reaction involving the C_{17}-OH group, characterized by the development of an absorption maximum at 235$\tilde{\upsilon}$ to 230$\tilde{\upsilon}$ and (3) intramolecular rearrangements. The use of diluted acid as a reagent favors the C_3 reaction. Subsequent dilution with water of a solution of α-estradiol in concentrated acid results in a reversible solvent effect and accelerates an irreversible rearrangement or reaction; this is characterized by the development of an absorption maximum at 207$\tilde{\upsilon}$ when the C_{17} group is involved, and at 195$\tilde{\upsilon}$ when the C_3 group is involved. Published results by Cohen and Bates, as well as by Umberger and Curtis, show that these processes are general for the estrogens. This explains the accurate timing, temperature, and concentration measurements required for the colorimetric assay of estrogens in acid solution. The reaction of α-estradiol with phenolsulphonic acid, and with phosphoric acid, involves only the C_3 group. Dehydrating reagents are necessary before an acid reaction involving the C_{17}—OH group can occur.

Introduction

This paper describes a series of experiments undertaken to determine the absorption and fluorescent properties of the compound formed by the reaction of sulphuric acid with α-estradiol. This work is part of a program designed to characterize a number of steroids, in a unique manner if possible, for use in the analysis of body fluids. The results are co-ordinated with those published by Cohen and Bates (2) and by Umberger and Curtis (5).

Methods

α-Estradiol was obtained from Ciba Company and was used without further purification. "Analar" sulphuric acid was used without further treatment, as the optical density of a 1.00 cm. thickness in the visible and ultraviolet region was consistently less than 0.04. Water was purified by distillation from alkaline potassium permanganate in an all-glass still, at a temperature below the boiling point.

Absorption measurements in the visible region were made on a photoelectric, spectrophotometer designed and built in these laboratories; the instrument will be described in a later publication. A Hilger Medium Quartz Spectrograph was used for absorption measurements in the ultraviolet region.

Fluorescent light was recorded photographically by placing a test tube of the solution under study at the slit of the Hilger Medium Quartz Spectrograph, which was at right angles to the incident light. The source of the

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incident light was a 100 watt G.E.C. A-H4 mercury discharge lamp, and a series of Baird interference filters; a Hanovia Sc. 2537 discharge lamp was also used as an ultraviolet source. Preliminary exposures showed no qualitative difference in the recorded bands when the slit was opened from 0.40 to 1.40 mm. The latter slit was therefore used together with exposure times of 30 min. on Kodak Super XX plates. The use of Eastman Type IV-0, ultraviolet sensitized spectrographic plates, cuts down the necessary exposure by a factor of 10.

The absorption measurements are reported in terms of the optical density D, where $D = \log_{10} I_0/I$, where I_0 = the intensity of the incident light and I = the intensity of the transmitted light, for a solution thickness of 1.00 cm. The spectral range is designated in wave number units, which are the reciprocals of the wave lengths measured in centimeters; the first three significant figures of the wave number are used throughout this paper and are indicated by the symbol ($\tilde{\upsilon}$). Wave numbers are the more fundamental analytical measurement, as they directly express the energy differences in a particular molecule, which give rise to its absorption and fluorescence properties.

Procedure and Results

Absorption

Amounts of the order of 100 μgm. of α-estradiol were obtained by evaporating aliquot portions of a standard solution in absolute ethanol. The steroid then readily dissolved in concentrated sulphuric acid to form a yellow solution. For convenience, this colored complex will be referred to as the conjugated form of the sterol. To determine the conditions necessary for the reaction between the acid and the α-estradiol to go to completion, a measured excess of acid was added to known weights of the steroid in test tubes, and the course of the reaction followed at 28° C., 57° C., and 100° C. by measuring the absorption of the solution at known intervals of time; the rates of change of

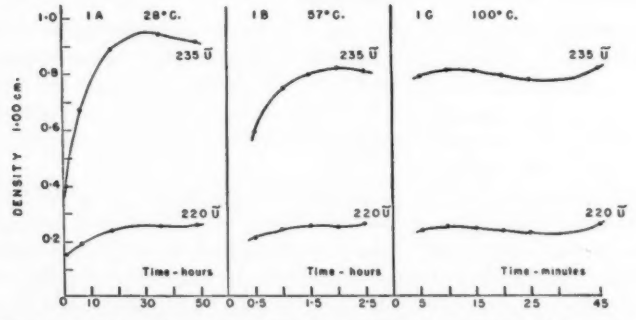

FIG. 1. Reaction rate curves of 183 γ α-estradiol dissolved in 20.0 ml. sulphuric acid.

the optical densities at 235$\tilde{\upsilon}$ and 220$\tilde{\upsilon}$ are shown in Fig. 1. A complete absorption curve of a solution obtained by dissolving α-estradiol in sulphuric acid by heating to 100° C. for 15 min. is given in Fig. 2. By varying the

volume of acid used, the applicability of Beer's Law to this particular solution was tested and the results are shown in Fig. 3. Identical results were obtained by diluting an initial solution of α-estradiol with cold sulphuric acid.

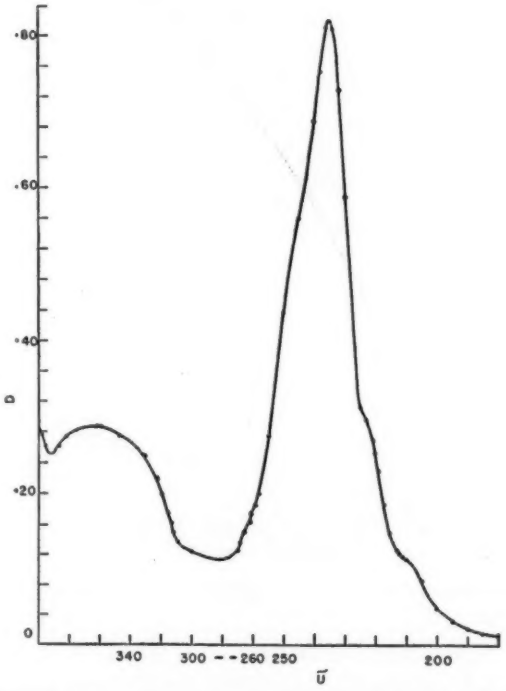

FIG. 2. Absorption curve of 183 γ α-estradiol dissolved in 20.0 ml. sulphuric acid at 100° C. for 15 min.

Experiments were then carried out to determine what effect a subsequent change in the solvent would have on the absorption properties of the steroid–acid complex formed in the concentrated sulphuric acid solution. Twelve samples of 200 γ of α-estradiol were dissolved in 2.0 ml. of concentrated sulphuric acid by heating at 100° C. for 15 min.; these solutions were cooled and diluted first with sulphuric acid, then with distilled water, to form a series of solutions containing 200 γ of α-estradiol in the conjugated form in 20.0 ml. of solvent. The solvents varied from concentrated sulphuric acid to 10% sulphuric in water by volume. Absorption measurements were made immediately after diluting the concentrated acid solutions.

The effects of increased proportions of water in the solvents were to bring about changes in the density and position of the maxima of absorption. These changes are listed in Table I. Examples of the type of change in the

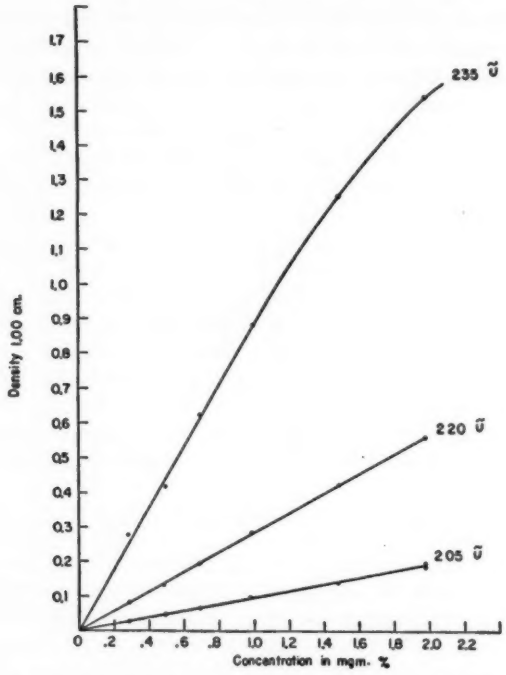

FIG. 3. Applicability of Beer's Law to α-estradiol in sulphuric acid at 100° C. for 15 min.

TABLE I

THE IMMEDIATE EFFECT OF DILUTING WITH WATER A SOLUTION OF α-ESTRADIOL IN CONCENTRATED SULPHURIC ACID.—200 γ IN 20.0 ML. OF FINAL SOLUTION

Concentrated H_2SO_4 % by volume	Density 1.00 cm. at wave numbers					
	235	233	232	231	230	207
100	.905					(.109)
80		1.04				(.157)
60			1.08			(.188)
50				1.01		(.184)
40					.966	(.188)
35					.932	(.188)
30					.732	.181
25					.552	.138
20					.486	.085
15					.143	.069
10					.066	.048

NOTE: *The bracketed numbers in this table distinguish the density value of 207 ῡ before the absorption peak is resolved.*

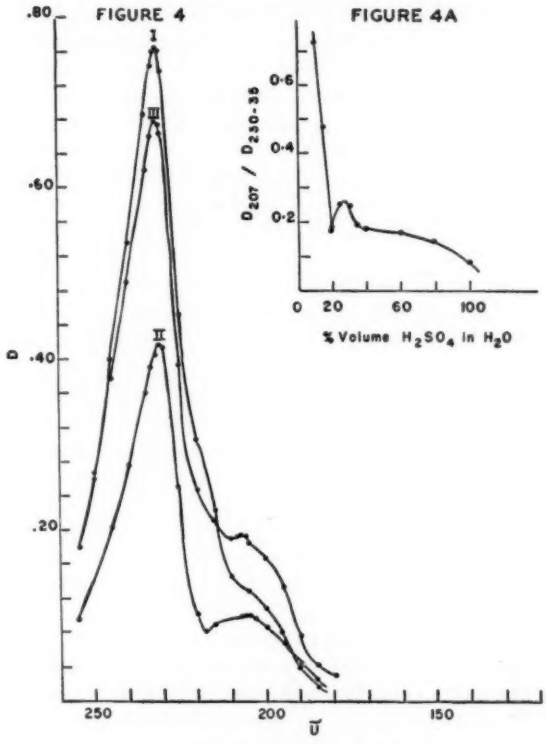

FIG. 4. Absorption curves 45γ α-estradiol in sulphuric acid.

I, 3.0 ml. sulphuric acid diluted with 3.0 ml. water;
II, 1.5 ml. sulphuric acid diluted with 4.5 ml. water;
III, 1.0 ml. sulphuric acid diluted with 3.0 ml. water followed by the immediate addition of 2.0 ml. sulphuric acid.

Fig. 4A. Ratio, density $207\tilde{\upsilon}$ to peak in $230\tilde{\upsilon}$–$235\tilde{\upsilon}$ band in diluted sulphuric acid solutions.

absorption curves, as a result of the change in the solvent, are shown in Fig. 4, Curves I and II, and in Fig. 4A.

Studies were carried out on the stability of the solutions listed in Table I. In concentrated sulphuric acid solution, the absorption curve of the steroid–acid conjugate increased only 2% in magnitude during 22 hr. at 28° C. Changes with time, of the absorption of the conjugate in certain acid–water solvents, were determined at 28° C., 57° C., and 100° C. These changes were qualitatively the same as those obtained by the progressive dilution shown in Table I. The density at $230\tilde{\upsilon}$ decreased, and a second maximum was resolved at $207\tilde{\upsilon}$. The density values obtained at these two wave numbers are listed in Table II, and a representative number of absorption

TABLE II

COURSE OF THE REACTION FOLLOWING THE DILUTION WITH WATER OF 45 γ α-ESTRADIOL IN CONCENTRATED ACID SOLUTION. TOTAL VOLUME OF ACID SOLUTION 6.0 ML.

Time, min.	Optical density reaction at 100° C.				Time, hr.	Optical density reaction at 57° C.	
	50% acid		25% acid			50%	acid
	230	207	230	207		230	207
3	.594	.247	—	—	0.5	.665	.193
10	.568	.280	.102	.090	2.0	.527	.216
20	.469	.289	.050	.072	3.0	.489	.224
30	.375	.258	.038	.065	3.75	.294	.286
					6.0	.247	.334

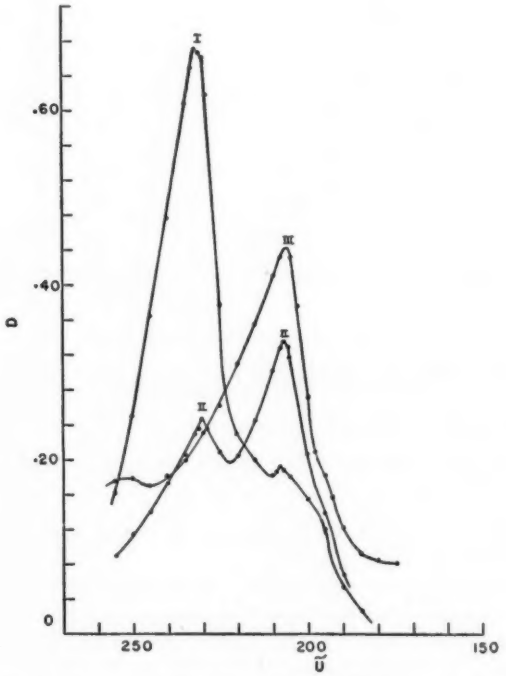

FIG. 5. Course of reaction initiated by adding 3.0 ml. water to 45γ α-estradiol in 3.0 ml. sulphuric acid.

I, after 5.5 hr. at 28° C.;

II, after 6 hr. at 57° C.;

III, after 15 min. at 100° C. followed by 16 hr. at 28° C.

curves are shown in Fig. 5. The effect of a different temperature, or a different acid concentration, was to alter the relative rates of density changes at 230$\tilde{u}$ and 207$\tilde{u}$.

These results indicated that a second reaction was initiated by the addition of water to the solution of conjugate in concentrated sulphuric acid. The course of the second reaction, characterized by the changes in the two absorption maxima, appeared to be compounded of two independent processes.

Further absorption measurements were carried out to demonstrate the dual nature of the reaction that followed the addition of water to the concentrated acid solution of the conjugate. Water was added to form a 25% by volume, acid–water solvent. After a measured interval of time, concentrated acid was added to readjust the solvent to 50% acid in water. The curves plotted in Fig. 4 compare the absorption of 50% solution (I), and a 25% solution (II), with the absorption of the solution prepared in 25% acid then readjusted to 50% acid (III). These results indicated that alteration of the solvent decreased the absorption density at 230$\tilde{u}$ by a reversible solvent effect; and concurrently an irreversible chemical reaction occurred, characterized by a nonreversible decrease in the 230$\tilde{u}$ maximum and resolution of the 207$\tilde{u}$ peak. As the extent of the chemical reaction was allowed to proceed by allowing the 25% solution (II) to age before readjusting to 50% again, the reversible effect decreased and disappeared when the 25% acid solution was allowed to reach the stage represented by a single absorption maximum at 207$\tilde{u}$.

Cohen and Bates (2) dissolved a number of estrogenic steroids in concentrated sulphuric acid, added a series of acid–water solvents, and heated the resulting solution at 100° C. for three minutes. Absorption densities calculated from their published absorption curves are summarized in Table III.

TABLE III *A*

OPTICAL DENSITIES AND SPECTRAL POSITIONS OF ABSORPTION MAXIMA, OBTAINED BY DILUTING A SOLUTION OF ESTROGEN IN CONCENTRATED SULPHURIC ACID WITH A DILUTE ACID SOLVENT, AND HEATING THE FINAL SOLUTION AT 100° C. FOR THREE MINUTES; FROM COHEN AND BATES (2). RESULTS CALCULATED TO 200 γ OF ESTROGEN IN 20.0 ML. OF SOLUTION

Estrogen	Density of 1.00 cm. in 45% H_2SO_4 at wave numbers		
	228	204	196
α-Estradiol	.348	.495	.482
Estrone			.782
Estriol			.691

The addition of the water solution to the α-estradiol conjugate under the conditions chosen has developed the absorption maxima very close to the wave numbers noted above in Table II and Fig. 5. In addition, the maximum at 196$\tilde{u}$ has been resolved.

TABLE III *B*

OPTICAL DENSITIES AND SPECTRAL POSITIONS OF ABSORPTION MAXIMA, OBTAINED BY DILUTING A SOLUTION OF ESTRONE IN CONCENTRATED SULPHURIC ACID WITH A SERIES OF DILUTE ACID SOLVENTS AND HEATING THE FINAL SOLUTION AT 100° C. FOR THREE MINUTES; FROM COHEN AND BATES (2). RESULTS CALCULATED TO 200 γ OF ESTRONE IN 20.0 ML. OF SOLUTION

Concentration H_2SO_4 % by volume	Density 1.00 cm. at wave numbers			
	221	220	218	196
91.5	1.34			
79.0		1.58		
58.0			1.58	
37.4			(.280)	.778
25.0			(.202)	.628
17.0			(.140)	.478

NOTE: *The bracketed numbers in this table distinguish the density values before the absorption peak is resolved.*

TABLE IV

OPTICAL DENSITIES AND SPECTRAL POSITIONS OF THE ABSORPTION MAXIMA OF ESTROGENS HEATED AT 100° C. FOR 12 MIN. IN A SERIES OF SULPHURIC ACID SOLUTIONS; FROM UMBERGER AND CURTIS (5). RESULTS CALCULATED TO 200 γ OF ESTROGEN IN 20.0 ML. OF FINAL SOLUTION

Concentration H_2SO_4, % by volume	Density 1.00 cm. at wave numbers												
	235	232	231	223	222	220	219	218	217	216	198	197	195
α-Estradiol													
100	.798				(.398)								
90		.860			.657								
75						.996							
60									.782			.459	
50*									.521				.375
45										.298			.169
β-Estradiol													
100			.250		.240								
90			(.320)		.463								
75							.942						
60								1.16					.320
45										.494			.927
30													.418
Estrone													
100				.668									
90					1.31								
75						1.37							
60							.816				.519		
45									.080				.120
Equilin													
100										.654			
90										.660			
75								.697					
60								.250			.259		

* *Measurements made in this laboratory (Fig. 6, I).*

An extensive series of experiments to obtain the absorption curves of α-estradiol and other estrogens in a series of dilute sulphuric acid solutions has been carried out by Umberger and Curtis (5). These authors diluted the acid with water before dissolving the estrogens, thus forming the conjugate in the diluted acid. Results calculated from the data reported by these workers are given in Table IV.

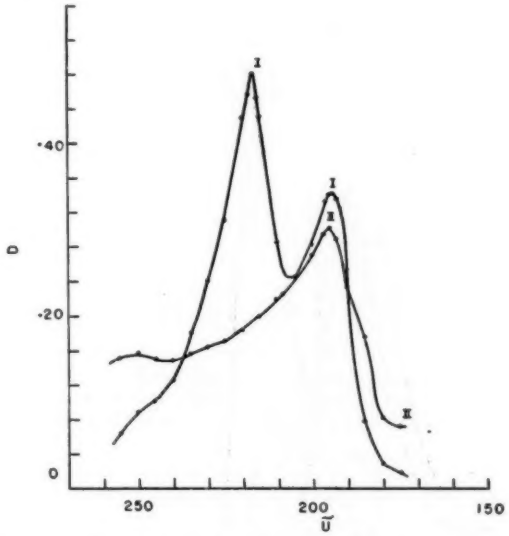

FIG. 6. I, 183γ α-Estradiol dissolved in 50% sulphuric acid and heated 12 min. at 100° C.;
II, Solution I heated another 15 min. at 100° C. and then 16 hr. at 28° C.

Experiments relating to the effect of the acid environment on the absorption properties of α-estradiol were extended to include other reagents already reported in the literature. The results are shown in Fig. 7. Jailer (3), and Bates and Cohen (1), have described solvents consisting of about 60% sulphuric acid in a water–alcohol mixture, designed to increase the fluorescence intensity of α-estradiol. These media produced the same absorption curves, Fig. 7, II. The effect of substituting 88% phosphoric acid for concentrated sulphuric acid as a solvent, is shown in Fig. 7, I. The absorption curve resulting from the use of Kober's phenolsulphonic acid reagent in the manner of Venning (6) is shown in Fig. 7, III.

Measurements of the absorption in the ultraviolet region, of the complex formed by α-estradiol and sulphuric acid, have not been as extensive as those in the visible, because the technique would be more cumbersome for assay purposes and no increase in absorption sensitivity was observed. Table V lists the wave number and density of the absorption maxima for a number of acid

solutions containing 200 γ of α-estradiol in 20.0 ml. of solvent. The three 50% solutions listed were measured (1) immediately after dilution of a concentrated acid solution of the conjugate; (2) following treatment of (1) at 100° C. for 20 min.; (3) following the use of 50% sulphuric acid as a reagent on the solid estradiol at 100° C. for 12 min. according to the method of Umberger and Curtis.

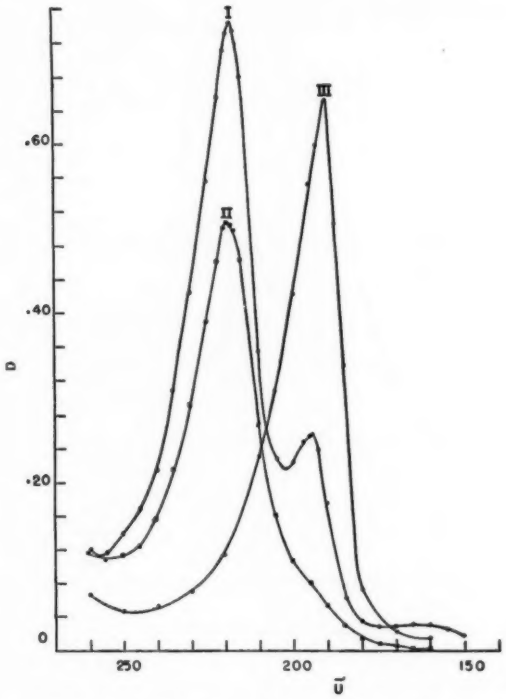

FIG. 7. I, 183γ α-Estradiol in 20.0 ml. phosphoric acid for 15 min. at 100° C.;
II, 49.5γ α-Estradiol in 8.5 ml. Jailer's solution;
III, 198γ α-Estradiol in 15.0 ml. Kober's reagent.

TABLE V

ABSORPTION OF α-ESTRADIOL EXTENDED TO ULTRAVIOLET REGION

Absorption of 200γ α-estradiol in 20.0 ml. acid solutions

Acid reagent	Density 1.00 cm. at following wave numbers							
	440	380	370	360	315	235	230	217
Concentrated H_2SO_4	.500			.305		.894		
50% H_2SO_4 immediately			.403				1.01	
50% H_2SO_4 100° C. 20 min.		.711			.410		.712	
50% H_2SO_4 as reagent			.109					.524

Procedure and Results

Fluorescence

The fluorescent bands emitted by α-estradiol in acid solutions were dependent upon the wave number of the incident exciting light, and upon the proportion of acid in the solvents. The bands recorded when the conjugate in concentrated sulphuric acid was irradiated by ultraviolet and visible light are given in Table VI. The effect of different solvents on the bands, emitted

TABLE VI

FLUORESCENCE OF 200 γ α-ESTRADIOL IN 20.0 ML. CONCENTRATED SULPHURIC ACID

Incident light	Fluorescent bands ($\tilde{\upsilon}$)				
394	227*	—	—	—	—
274	227	215	—	—	—
247	227	218	191	176	—
229	226	215	191	176	—
183	—	—	—	—	166

* *Under the action of 394$\tilde{\upsilon}$ incident light photochemical bleaching of the solution occurred.*

TABLE VII *A*

FLUORESCENCE OF α-ESTRADIOL SOLUTIONS. SOLUTIONS MADE UP OF 200 γ OF α-ESTRADIOL IN 20.0 ML. SOLVENT. INCIDENT LIGHT 274$\tilde{\upsilon}$

Solvent % H_2SO_4 in H_2O by volume	Fluorescent bands ($\tilde{\upsilon}$)		
100	—	227	215
75	—	225	213
50	—	224	213
40	—	222	211
30	—	222	215
25	252	222	—
20	251	220	—
15	250	220	—
10	250	220	—
1	—	—	—

TABLE VII *B*

FLUORESCENCE OF α-ESTRADIOL SOLUTIONS. SOLUTIONS MADE UP OF 200 γ OF α-ESTRADIOL IN 20.0 ML. SOLVENT. INCIDENT LIGHT 229$\tilde{\upsilon}$

Solvent % H_2SO_4 in H_2O by volume	Fluorescent bands ($\tilde{\upsilon}$)			
100	226	215	191	176
75	224	212	191	174
50	222	211	191	174
35	220	208	191	173
25	220	209	—	173
10	219	—	—	—
1	—	—	—	173

under light of a given wave number, is shown in Tables VII *A* and VII *B*. The bands were approximately 10$\tilde{\upsilon}$ in width; the positions of the band centers are plotted in these tables.

Discussion

The data of Tables I, III, and IV may be readily systematized and explained by relating the absorption maxima to the —OH groups present as in Table VIII below:

TABLE VIII

Chemical grouping	Wave number of absorption maxima	
	Concentrated acid solution	Dilute acid solution
C_{17}—OH	235$\tilde{\upsilon}$ to 230$\tilde{\upsilon}$	207$\tilde{\upsilon}$ to 204$\tilde{\upsilon}$
C_3—OH	223$\tilde{\upsilon}$ to 220$\tilde{\upsilon}$	198$\tilde{\upsilon}$ to 195$\tilde{\upsilon}$

When the steroid conjugates are formed in concentrated sulphuric acid, and the resulting solutions are then diluted with water, the absorption maxima in the dilute acid solutions develop at the expense of the maxima in the concentrated acid. The rate of this rearrangement depends upon the proportion of water, the temperature, and the particular estrogen conjugate in the solution. In the concentrated acid solutions, α-estradiol has a prominent absorption maximum at 235$\tilde{\upsilon}$ due to the C_{17}—OH group, and an inflection (Fig. 1), at 220$\tilde{\upsilon}$ due to the C_3—OH group. Increasing proportions of water added to this solution first alters the peak position from 235$\tilde{\upsilon}$ to 230$\tilde{\upsilon}$, accompanied by an increase in optical density; as the acid concentration is reduced below 40% at room temperature, the 207$\tilde{\upsilon}$ maximum is resolved, accompanied by a decrease in the density at 230$\tilde{\upsilon}$ and the disappearance of the inflection at 220$\tilde{\upsilon}$, Table I and Fig. 4.

Estrone in concentrated acid solution has an absorption maximum at 223$\tilde{\upsilon}$. Increasing proportions of water added to this solution first alters this peak to 218$\tilde{\upsilon}$ accompanied by an increase in optical density. As the acid concentration is reduced below 45%, at 100° C., the 196$\tilde{\upsilon}$ maximum is resolved, accompanied by the disappearance of the maximum at 218$\tilde{\upsilon}$ (Table III *B*.)

Absorption maxima at 196$\tilde{\upsilon}$, characteristic of the C_3—OH group, appear when solutions of α-estradiol and estriol conjugate, in concentrated acid, are diluted to 45% acid at 100° C. The single maxima at 196$\tilde{\upsilon}$ distinguishes estriol and estrone from α-estradiol (Table III *A*.)

The formation of the steroid–acid conjugate in diluted acid favors the reaction involving the C_3—OH group. In Table IV, it is shown that only the 220$\tilde{\upsilon}$ maxima occur when α-estradiol is dissolved in 75% acid, and when β-estradiol is dissolved in 90% acid. The absorption maxima of the estrone

solution in Table IV are similar to those recorded in Table III *B*; the use of the diluted acid reagent, does, however, permit the resolution of the 196$\tilde{u}$ maximum at stronger acid concentrations.

Further points of interest in Table IV may be noticed. Comparing the two forms of estradiol, the higher reactivity of the β-orientated C_{17}—OH group may explain the lower absorption maximum at 231$\tilde{u}$ in concentrated acid. The comparison of the two forms of estradiol also suggests that the shift of the absorption maximum from 235$\tilde{u}$ to 230$\tilde{u}$ when a solution of α-estradiol in concentrated sulphuric acid is diluted with water (Table I) is due to a reorientation of the conjugate associated with the C_{17} atom. Again from Table IV the shift of the absorption maximum from 223$\tilde{u}$, in the case of estrone, to 216$\tilde{u}$ in the case of equilin, with the occurrence of an additional double bond in Ring *B*, tends to confirm the association of the 220$\tilde{u}$ absorption region with Ring *A* of the estrogens.

It is of interest to note in Table V, that the absorption maximum in the ultraviolet region, of α-estradiol in 50% acid solution is at the same wave number regardless of whether the steroid is first dissolved in concentrated acid to produce an absorption maximum at 230$\tilde{u}$, characteristic of the C_{17}—OH group, or whether it is dissolved in the diluted acid to produce an absorption maximum at 220$\tilde{u}$ characteristic of the C_3—OH group.

The absorption maxima obtained by dissolving α-estradiol in the different acid reagents are given in Table IX below:

TABLE IX

ABSORPTION MAXIMA OF α-ESTRADIOL IN ACID SOLUTIONS

Acid reagent	Maximum density 1.00 cm. of 200 γ in 20.0 ml.				
	232	220	207	195	191
Concentrated H_2SO_4	1.09		.181		
Diluted H_2SO_4		.545		.250	
88% H_3PO_4		.810		.279	
Phenolsulphonic acid					.722
Jailer's reagent		.872			

These results show that the C_{17} atom is affected only by concentrated sulphuric acid. The C_3 atom is affected by the remaining acid reagents, all of which contain water; the optical density distribution between 220$\tilde{u}$ and 195$\tilde{u}$ varies with the reagent.

The reaction rate curves of Fig. 1 may be explained by postulating the occurrence of three reactions when α-estradiol is dissolved in concentrated sulphuric acid:

(*a*) Sulphuric acid attacks the C_3—OH group at a rate represented by the more rapid approach to a maximum of the absorption density at 220$\tilde{u}$.

(*b*) Sulphuric acid attacks the C_{17}—OH group at a rate represented by the slower attainment of a maximum of absorption at 235$\tilde{\upsilon}$.

(*c*) Intramolecular rearrangements take place around the C_{17} atom represented by the slow subsequent decrease in absorption density at 235$\tilde{\upsilon}$ and by the lower maximum density attained at higher temperatures.

The explanation of the absorption phenomena in terms of the chemical formulae requires further studies. In this connection Miescher (4) has shown that colored compounds result from the addition of strong acids to organic systems containing at least one double bond and has suggested that a carbonium ion is formed. He has also indicated that color reactions involving the C_{17}—OH group require the initial removal of the hydroxyl group as water.

Fluorescence

The fluorescent bands occur as a result of the re-emission of absorbed light. In consequence they depend upon the absorption properties of the solution and upon the intensity and quality of the incident light. In all the α-estradiol solutions examined, the fluorescent light was of lower wave number than the absorbed light.

The most intense fluorescent bands are those at 227$\tilde{\upsilon}$ and 215$\tilde{\upsilon}$; the band at 227$\tilde{\upsilon}$ is common to all the solutions of α-estradiol in sulphuric acid and is responsible for the characteristic blue fluorescence in daylight.

The discontinuity in the fluorescence at 30% acid concentration under 274$\tilde{\upsilon}$ incident light, shown in Table VII *A*, corresponds to a discontinuity in the absorption properties at this acid concentration as shown in Fig. 4 *A*.

Acknowledgments

The author wishes to thank P. A. Macdonald, Director of the Manitoba Cancer Institute, for indicating the use of radiation measurements in the development of methods of hormone assay, and for the development of some of the optical equipment used in these researches.

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We would also express our grateful thanks to the Trustees of the War Fund of the Brewers and Hotelkeepers of Manitoba, for the financial assistance which enabled this work to be undertaken.

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THE ABSORPTION AND FLUORESCENCE PROPERTIES, IN THE VISIBLE SPECTRAL REGION, OF CERTAIN STEROIDS IN SULPHURIC ACID SOLUTIONS

By J. H. Linford and O. Bernice Paulson

Abstract

The absorption and fluorescence of steroids dissolved in concentrated sulphuric acid, and the changes in these properties on adding ethanol to the concentrated acid solutions, have been examined for: cholesterol, pregnandiol, α-estradiol, dehydroisoandrosterone (D.H.A.), dehydroisoandrosterone acetate, desoxycorticosterone acetate, (D.O.C.A.), testosterone propionate, and progesterone. At 57° C. or less, in concentrated sulphuric acid solution a colored compound was formed only if an hydroxyl group was present in the steroid molecule; the keto group formed a colored compound with sulphuric acid only in the presence of a hydroxyl group in the same steroid molecule. As the concentrated sulphuric acid solutions were diluted with alcohol, absorption maxima appeared which were characteristic of specific hydroxyl and keto groups. Irradiation of the solutions by light of the spectral regions corresponding to the absorption maxima produced related fluorescent bands.

Introduction

It has been shown in the visible spectral region that the absorption and fluorescence properties of estrogenic steroids, in sulphuric acid solutions, are dependent upon the positions of the hydroxyl groups (1). Further studies have been made on the following alcoholic and keto steroids: cholesterol, pregnandiol, α-estradiol, dehydroisoandrosterone (D.H.A.), dehydroisoandrosterone acetate, desoxycorticosterone acetate (D.O.C.A.), testosterone propionate, and progesterone. The present paper describes the absorption and fluorescence of these steroids in solution in concentrated sulphuric acid, and the changes in these properties on adding ethanol to the concentrated acid solutions. The object of this work was to characterize the steroids so they might be identified and measured in simple mixtures.

Methods and Procedure

Amounts of the order of 100 μgm. of each steroid were obtained by preparing solutions in absolute ethanol and evaporating aliquot portions in test tubes in a boiling water bath. The reagents used to prepare the colored steroid derivatives were "Analar" sulphuric acid, with no further purification, and absolute ethanol distilled once in glass without boiling. The absorption of the colored compounds formed was measured with a photoelectric spectrophotometer, designed and built in these laboratories. Fluorescent spectra were recorded by a Hilger Medium Quartz spectrograph; the incident light source used to excite fluorescence consisted of a G.E.C. AH-4 100 watt mercury discharge lamp and a series of Baird interference filters. Some experiments were carried out using the Hanovia Sc. 2537 arc, which emitted 80% 2537 Å

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radiation. The other 20% was distributed throughout the ultraviolet and visible region, but the slit width and exposure time were such that no appreciable fluorescence could be recorded from this radiation.

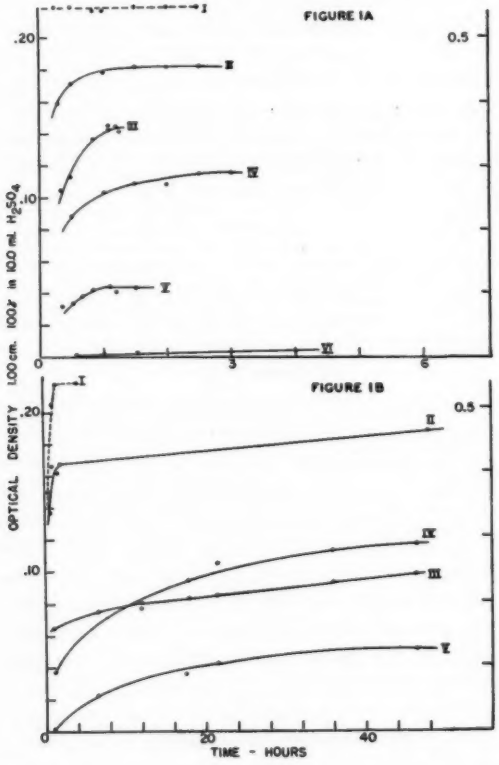

FIG. 1A. Rate of change of absorption maxima when steroids dissolved in concentrated sulphuric acid at 57° C.

I, D.H.A. and its acetate (scale on right);
II, pregnandiol;
III, D.O.C.A.;
IV, cholesterol;
V, testosterone;
VI, progesterone.

FIG. 1B. Rate of change of absorption maxima when steroids dissolved in concentrated sulphuric acid at 28° C.

I, D.H.A. (scale on right);
II, pregnandiol;
III, D.O.C.A.;
IV, cholesterol;
V, testosterone.

The absorption measurements are reported in terms of the optical density D, where $D = \log_{10} I_0/I$, for a solution thickness of 1.00 cm. The spectral range is designated in wave number units, which are the reciprocals of the wave lengths measured in centimeters; the first three significant figures of the wave number are used throughout this paper and are indicated by the symbol ($\tilde{\upsilon}$). Wave numbers are the more fundamental analytical measurement, as they express the energy differences in a particular molecule, which give rise to its absorption and fluorescence properties.

To determine the conditions necessary for the reaction of the sulphuric acid on the steroids to go to completion, series of reaction rate measurements were made at 28° C. and at 57° C. The reactions, resulting from the addition of concentrated sulphuric acid to the solid steroids at these temperatures, were followed by determining the absorption curves at measured intervals of time, each curve being measured on a separate solution. The results are shown in Fig 1*A* and 1*B*, where the value of the maximum optical density is plotted against time in hours.

A study of the absorption and fluorescent properties was then carried out on samples of the steroids dissolved in concentrated sulphuric acid by heating at 57° C. for one hour and then diluting with absolute ethanol such that, for each steroid, a series of solutions of the same concentration was obtained, the solvents being 100%, 75%, 50%, 25%, and 10%, sulphuric acid in ethanol by volume. In the previous work on α-estradiol, water was used as the diluent, but this produced precipitates in a number of the steroid–acid solutions. Alcohol dilution was therefore used throughout the present work, the acid solutions being cooled in ice water during the slow addition of the alcohol.

The applicability of Beer's Law was examined for each steroid in sulphuric acid solution. A linear relationship between concentrations of steroid and optical density of a 1.00 cm. thickness of the solution was found to hold for cholesterol, pregnandiol, D.O.C.A., and testosterone, to a density of 1.5, which is the limit that can be accurately measured on the visible spectrophotometer. Deviations from linearity below this limit were found in the case of α-estradiol, for which the $235\tilde{\upsilon}$ maximum was nonlinear above 1.0 mgm. % concentration (D, 1.00 cm. = 0.9). Possibly in the case of D.H.A., the maximum would be nonlinear above 3.0 mgm. % (D, 1.00 cm. = 1.5).

Results and Discussion

The absorption curves of the steroids in concentrated sulphuric acid solution, after one hour at 57° C. are shown in Fig. 2. The absorption curves resulting from the addition of a measured volume of ethanol to these solutions in concentrated acid are plotted in Figs. 3–9. Graphs which show the wave number limits of the fluorescence bands as recorded photographically, and which give qualitative indication of their relative intensities, are plotted in Figs. 10–16. The incident light frequency is represented by a narrow rectangle, the fluorescent bands by triangles of which the peak indicates the densest portion. For each steroid a table is compiled describing the appearance of each solution

to the eye in daylight and the fluorescent colors discernible to the eye in a room darkened but for the incident light. The intense fluorescent colors in each table are marked with an asterisk (Tables IV–X).

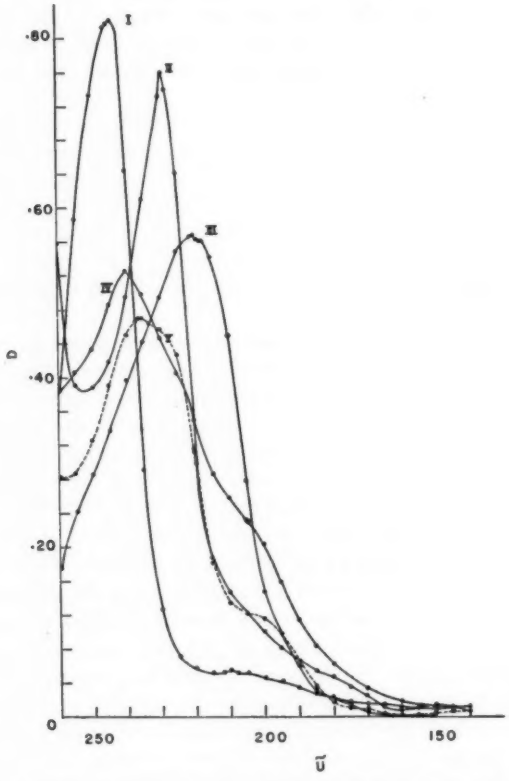

FIG. 2. Absorption curves of steroids dissolved in concentrated sulphuric acid.

I, dehydroisoandrosterone—180 γ per 12 ml.;
II, desoxycorticosterone acetate—161 γ per 2 ml.;
III, testosterone propionate—303 γ per 2 ml.;
IV, cholesterol—215 γ per 4 ml.;
V, pregnandiol—122 γ per 4 ml.

Absorption

In concentrated sulphuric acid solution the sterols differed from each other in the positions of the absorption maxima, but the differences in position were small and all solutions were yellow in color. The results obtained from dehydroisoandrosterone were identical with those obtained from its acetate ester calculated to the same weight of sterol. No visible color formed in the progesterone solutions.

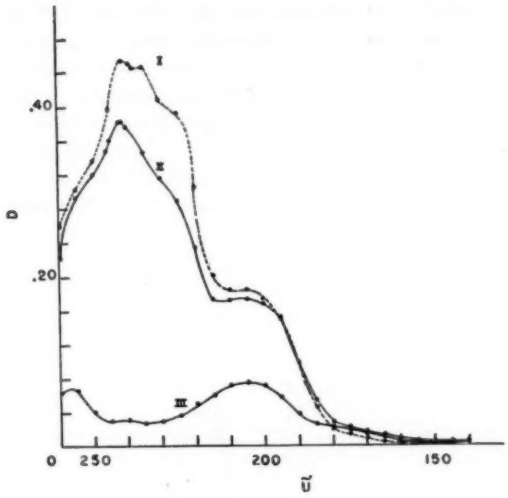

FIG. 3. Pregnandiol—122 γ in 4 ml. solution.

I, 75% sulphuric acid in ethanol;
II, 50% sulphuric acid in ethanol;
III, 25% sulphuric acid in ethanol.

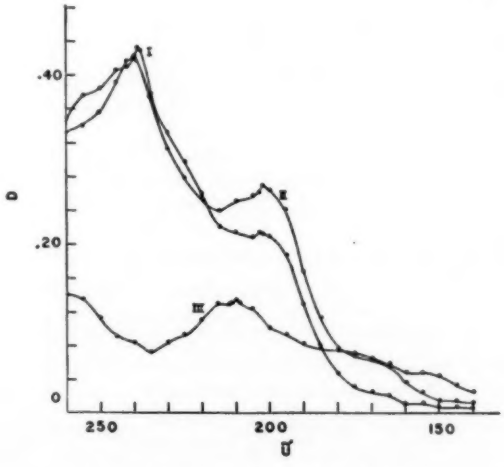

FIG. 4. Cholesterol—215 γ in 4 ml. solution.

I, 75% sulphuric acid in ethanol;
II, 50% sulphuric acid in ethanol;
III, 25% sulphuric acid in ethanol.

The progressive addition of ethanol to the concentrated acid solutions markedly altered the positions of the absorption maxima as follows:

Pregnandiol (Fig. 3) showed a decrease in intensity of the maximum at 240$\tilde{\upsilon}$ coupled with the appearance of a second maximum at 230$\tilde{\upsilon}$; cholesterol (Fig. 4) showed a decrease in the maximum at 240$\tilde{\upsilon}$ coupled with the resolution of a maximum at 210$\tilde{\upsilon}$.

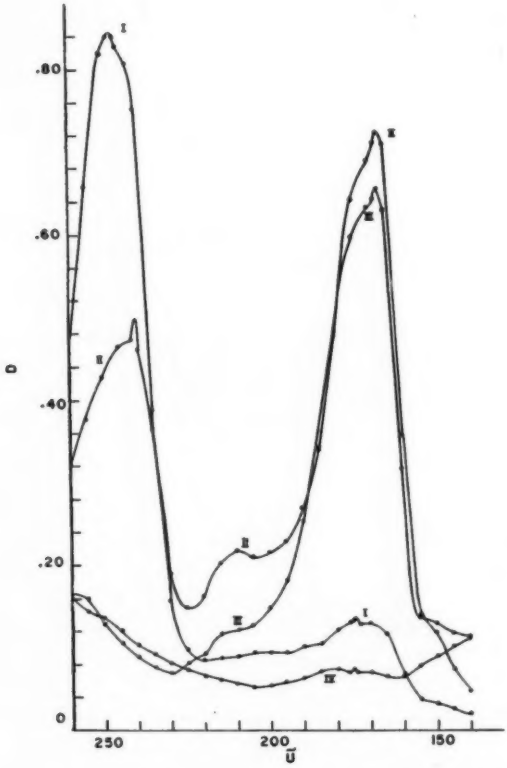

FIG. 5. Dehydroisoandrosterone—180 γ in 8 ml. solution.

I, 75% sulphuric acid in ethanol;
II, 50% sulphuric acid in ethanol;
III, 25% sulphuric acid in ethanol;
IV, 10% sulphuric acid in ethanol.

The curve of D.H.A. (Fig. 5) in 75% alcohol differed from the curve in concentrated sulphuric acid only by the slight increase at 170$\tilde{\upsilon}$. The further increase in the absorption intensity at this wave number, on decreasing the

acid content to 50% and 25% was so great that the solution became an intense blue color. During the progressive dilution of the acid the absorption maximum at 210$\tilde{\upsilon}$ became prominent and disappeared again, and the absorption maximum, initially at 245$\tilde{\upsilon}$, shifted to 240$\tilde{\upsilon}$ at 50% acid concentration.

The absorption curves of desoxycorticosterone acetate in acid–alcohol mixtures (Fig. 6) showed not only a marked absorption increase at 168$\tilde{\upsilon}$ as the proportion of acid was decreased but the intensity of the absorption was increased fivefold over the concentrated sulphuric acid solution. With decrease

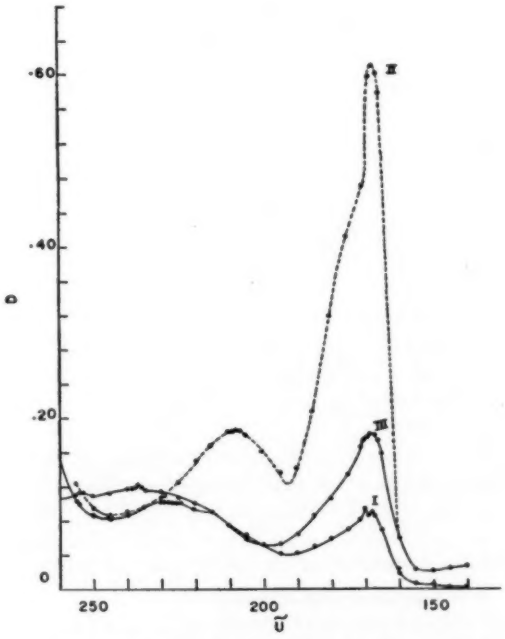

FIG. 6. Desoxycorticosterone acetate 161 γ in 16.0 ml.

I, 75% sulphuric acid in ethanol;
II, 50% sulphuric acid in ethanol;
III, 10% sulphuric acid in ethanol;
25% sulphuric acid in ethanol (not plotted), similar to II.

of the acid concentration, there was a decrease in the initial absorption maximum at 230$\tilde{\upsilon}$. The transient appearance of the 210$\tilde{\upsilon}$ peak, and the intense blue color of the solutions were similar to the phenomena observed with dehydroisoandrosterone.

The absorption peak of testosterone in concentrated sulphuric acid was at 220$\tilde{\upsilon}$, the lowest wave number for the steroids studied. As can be seen from

Fig. 7, the effect of dilution was not only to produce a marked increase in absorption intensity at 170$\tilde{\upsilon}$, similar to that observed with the two keto steroids already described, but also an equal increase in the 205$\tilde{\upsilon}$ maximum.

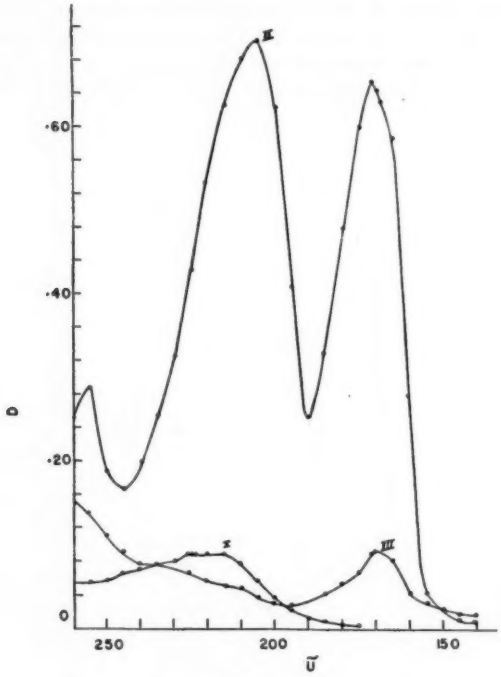

FIG. 7. Testosterone propionate 303 γ in 16.0 ml.

I, 75% sulphuric acid in ethanol;
II, 50% sulphuric acid in ethanol;
III, 10% sulphuric acid in ethanol;
25% sulphuric acid in ethanol, is similar to II.

The resulting color of the acid–alcohol solution was therefore green. Again, the absorption intensities were more than five times that of the concentrated sulphuric acid solution.

Among the steroids studied, progesterone has been unique in that it readily dissolved in concentrated sulphuric acid in the cold, but no color was evident after prolonged time at room temperature. When heated for three hours at 57°C., the acid solutions remained colorless unless the steroid concentration was very high, when a yellow color was discernible. On diluting the colorless acid solutions with alcohol, however, a faint color appeared. Heating the concentrated acid solution at 100° C. for 15 min. produced a yellow color of

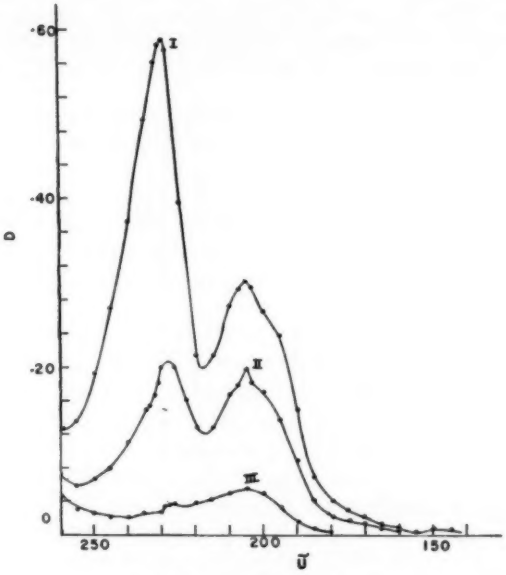

FIG. 8. Estradiol 99 γ in 10.0 ml.

I, 40% sulphuric acid in ethanol;
II, 30% sulphuric acid in ethanol;
III, 20% sulphuric acid in ethanol.

the same order of absorption intensity as testosterone (Fig. 9). Addition of alcohol to this yellow solution produced a marked increase of absorption at 210ṽ, and 166ṽ, very like that observed with testosterone.

The absorption results obtained from the 50% acid in alcohol solutions are collected in Table I.

TABLE I

Steroid	Spectral positions and the optical densities of the absorption maxima of 100γ of steroid dissolved in 5.0 ml. conc. H_2SO_4 immediately following the addition to this solution of 5.0 ml. absolute ethanol					
	240	230	220	210	205	170
Pregnandiol	.184	.105			.057	
Cholesterol	.078				.052	
D.H.A.	.222			.096		.321
D.O.C.A.		.100		.186		.607
Testosterone					.372	.347
Progesterone (100°C.)					.234	.310
α-Estradiol		1.00			.300	
Estrone			1.58			

A study of this table reveals that the alteration of the solvent results in the absorption of light of wave numbers characteristic of definite chemical groups in the molecule. The presence of the —OH group at the C_3 position of a

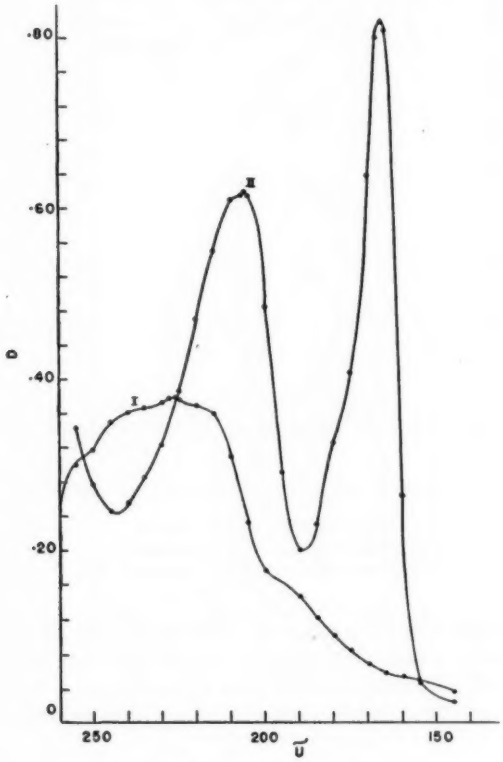

FIG. 9. Progesterone.

I, 106 γ in 2.0 ml. sulphuric acid heated 100° C. for 30 min.
II, Solution I diluted with 2.0 ml. ethanol.

saturated *A* ring corresponds to absorption at 240$\tilde{\upsilon}$; the presence of the —OH group at the C_{17} position or in a C_{17} side chain corresponds to absorption at 230$\tilde{\upsilon}$. The presence of a keto group corresponds to absorption 166$\tilde{\upsilon}$ to 170$\tilde{\upsilon}$. To the table has been added a result (estrone) from the previous paper (1) where in acid–water solutions the association of the C_{17}—OH group of the estradiols with absorption at 230$\tilde{\upsilon}$ was also demonstrated, and where the phenolic C_3—OH group was shown to cause an absorption peak at 220$\tilde{\upsilon}$; the latter has also been confirmed in acid–alcohol solutions. Testosterone appears to be an exception to this scheme as no absorption maximum at

230$\tilde{\upsilon}$, associated with the C_{17}—OH group, is evident. This may be masked by the high absorption at 220$\tilde{\upsilon}$ in concentrated acid and at 205$\tilde{\upsilon}$ in the acid alcohol solutions.

The relationship of the 205–210$\tilde{\upsilon}$ absorption maximum to the constitution of the molecule is more difficult to find. In the previous paper (1) the development of a 205$\tilde{\upsilon}$ maximum was shown to be associated with the diminution of the 230$\tilde{\upsilon}$ peak, and the high intensity of absorption at 205$\tilde{\upsilon}$ in the case of testosterone may explain the absence of a maximum at 230$\tilde{\upsilon}$. However, the 205$\tilde{\upsilon}$ and 210$\tilde{\upsilon}$ maxima are present in the cases of cholesterol and D.H.A. which contain a C_3 alcoholic —OH group; these peaks may signify any alcoholic —OH group.

The results obtained by heating progesterone in acid to 100° C. indicate that the presence of the 220$\tilde{\upsilon}$ absorption maximum when testosterone and progesterone are dissolved in concentrated sulphuric acid may be caused by the conjugated keto group. D.O.C.A., which has this grouping, shows evidence of an absorption maximum at 220$\tilde{\upsilon}$ in 75% acid solution. The absorption peaks at 166–170$\tilde{\upsilon}$, which appear when alcohol is added therefore result from the absorption at 220$\tilde{\upsilon}$ in the concentrated acid solutions. The absorption around 220$\tilde{\upsilon}$ associated with the phenolic —OH group is readily differentiated by its secondary maximum at 196$\tilde{\upsilon}$ (1).

Fluorescence

The fluorescence phenomena recorded in Figs. 10–16 showed that the highest intensity of fluorescence coupled with the largest number of bands, for each

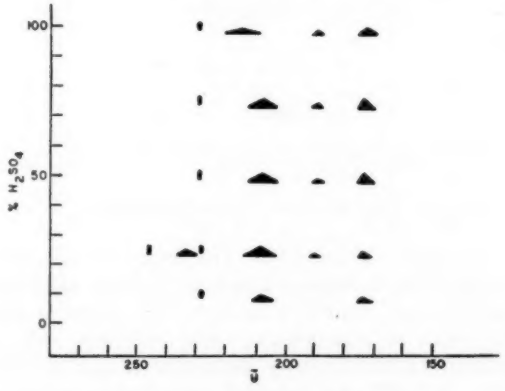

FIG. 10. Fluorescence of cholesterol solutions.

solution, were produced by using blue (229$\tilde{\upsilon}$) incident radiation. The wave number positions of the centers of the bands obtained from 50% acid in alcohol solution are listed in Table II.

TABLE II

Steroid	Fluorescence bands of 50% acid solutions in alcohol under 229$\tilde{\upsilon}$ radiation $\tilde{\upsilon}$						
Pregnandiol			210	190	175		
Cholesterol			210	190	175		
D.H.A.		215		190		155	
D.O.C.A.		215		190		155	
Testosterone				190	175	155	145
Progesterone				190	175	155	
α-Estradiol (not shown on graphs)	220		210	190	175		

Some spectrographic records were made on the effect of the wave number, of the incident light. The use of 274$\tilde{\upsilon}$ exciting light permitted additional fluorescent bands of wave number 230–250$\tilde{\upsilon}$ and also in some cases simplified

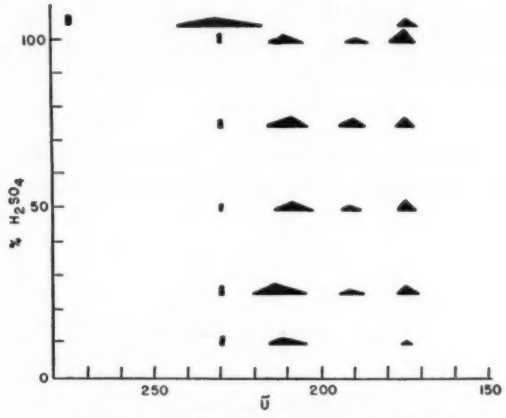

FIG. 11. Fluorescence of pregnandiol solutions.

the band system (see Figs. 11 and 14). The effect of radiation by 394$\tilde{\upsilon}$ (2537 Å) light, for the steroids dissolved in concentrated sulphuric acid and in 25% acid in alcohol, was examined using as the source of radiation the Hanovia SC 2537 arc. In concentrated acid solution, only α-estradiol fluoresced; a blue band at 227$\tilde{\upsilon}$ sharply distinguished this steroid from the other six examined. In 25% acid solution, α-estradiol emitted a band at 250$\tilde{\upsilon}$; testosterone fluoresced strongly, emitting two bands in the red region at 157$\tilde{\upsilon}$ and 147$\tilde{\upsilon}$; progesterone and D.O.C.A. fluoresced at 160$\tilde{\upsilon}$, the intensity being much less than that of testosterone; the progesterone solution was prepared by heating the initial solution in concentrated acid to 100° C. for 15 min.

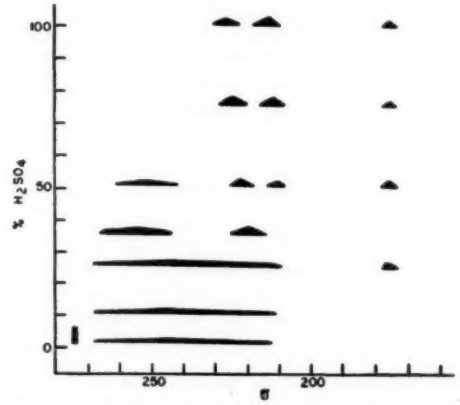

FIG. 12. Fluorescence of α-estradiol in sulphuric acid – ethanol solutions. Incident light 274×10^3 cm.$^{-1}$ = 3650 Å.

Radiation by green light (183$\tilde{\upsilon}$) permitted only the yellow and red fluorescence (160–175$\tilde{\upsilon}$) to occur (see Fig. 13). Radiation by yellow light (173$\tilde{\upsilon}$) permitted only the red fluorescence (160$\tilde{\upsilon}$) to occur (see Fig. 16).

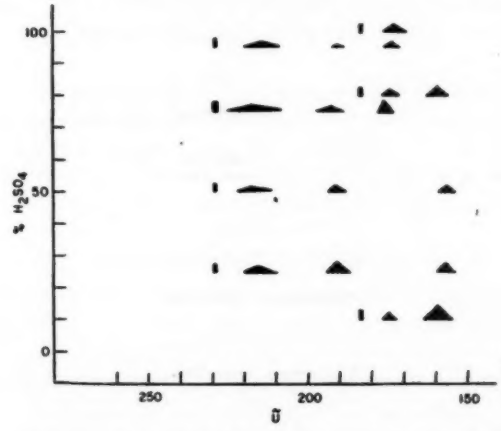

FIG. 13. Fluorescence of dehydroisoandrosterone solutions.

The effect of the wave number of the incident light on the fluorescent bands obtained from testosterone in 25% acid in alcohol solution is summarized in Table III.

The highest frequency of fluorescent light observed in the present study was that of α-estradiol at 250$\tilde{\upsilon}$ in 25% acid solution under 394$\tilde{\upsilon}$ radiation: thus all fluorescence was restricted to the visible spectral region.

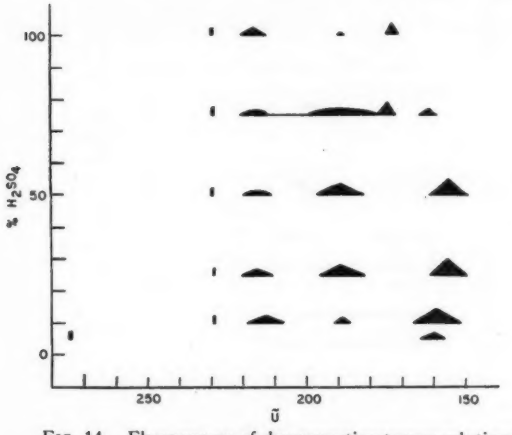

FIG. 14. Fluorescence of desoxycorticosterone solutions.

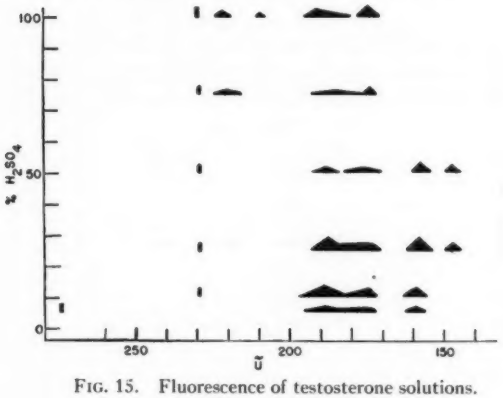

FIG. 15. Fluorescence of testosterone solutions.

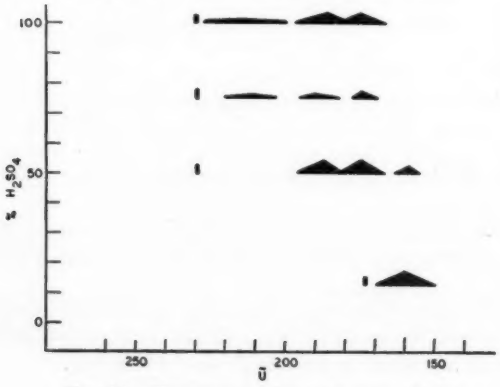

FIG. 16. Fluorescence of progesterone solutions.

TABLE III

FLUORESCENCE OF TESTOSTERONE PROPIONATE IN 25% ACID IN ALCOHOL

Incident light $\tilde{\upsilon}$	Fluorescent bands $\tilde{\upsilon}$		
394		157	147
274	185	157	147
229	185	157	147
183		157	147

Certain correlations between the fluorescence bands and absorption maxima at higher wave numbers were evident from a study of the above results. The $160\tilde{\upsilon}$ fluorescence band characteristic of the keto steroids occurred in conjunction with their absorption maxima at $170\tilde{\upsilon}$ and was of maximum intensity under incident radiation of that spectral region. Of the steroids studied, only testosterone and progesterone lacked a fluorescence band in the 210–$220\tilde{\upsilon}$ region under $229\tilde{\upsilon}$ incident light; these steroids had no absorption maxima in that region; on the other hand α-estradiol, which has a strong absorption maximum at $230\tilde{\upsilon}$ showed the strongest 210–$220\tilde{\upsilon}$ fluorescence under $229\tilde{\upsilon}$ incident light. The appearance of fluorescence bands around $230\tilde{\upsilon}$ followed the absorption of light of $274\tilde{\upsilon}$, indicating the presence of absorption maxima in that region.

The differences in fluorescent light when the solvent and incident radiation were varied were sufficiently marked to distinguish many of the steroids by simple inspection using only the arc and filters as equipment. For this reason the fluorescing colors are described in Tables IV to X.

TABLE IV

CHOLESTEROL FLUORESCENCE

Incident light, $\tilde{\upsilon}$	Concentration of acid solution in alcohol (v/v)			
	100%	50%	25%	10%
274	Bluish-white	Light blue	Light blue	Blue*
229	Yellow-green	Yellow-green*	Pale yellow-green	Faint yellow-green
183	Pale orange	Orange*	Pale orange	- - - - -
173	Pale orange	Pale orange	Pale orange	- - - - -
Solution color in daylight	Yellow	Pale yellow	Colorless	Colorless

* *Intense fluorescent color.*

TABLE V

PREGNANDIOL FLUORESCENCE

Incident light, $\tilde{\upsilon}$	Concentration of acid solution in alcohol (v/v)			
	100%	50%	25%	10%
274	Light blue	Light blue	Blue	Blue
229	Yellow-green*	Yellow-green*	Yellow-green*	Fainter yellow-green
183	Orange	Pale orange	Pale orange	Pale orange
173	Faint orange	Faint orange	Faint orange	Faint orange
Solution color in daylight	Yellow	Pale yellow	Colorless	Colorless

* *Intense fluorescent color.*

TABLE VI

α-ESTRADIOL FLUORESCENCE

Incident light, $\tilde{\upsilon}$	Concentration of acid solution in alcohol (v/v)			
	100%	50%	25%	10%
274	Blue*	Green*	Blue-green*	Blue
229	Blue*	Blue*	Blue-green*	Green
183	- - - - -	Orange	Light orange	- - - - -
173	- - - - -	- - - - -	- - - - -	- - - - -
Solution color in daylight	Yellow with green fl.	Yellow with green fl.	Yellow with green fl.	Colorless

* *Intense fluorescent color.*

TABLE VII

DEHYDROISOANDROSTERONE FLUORESCENCE

Incident light, $\tilde{\upsilon}$	Concentration of acid solution in alcohol (v/v)			
	100%	50%	25%	10%
274	Pale blue	Bluish-white	Greenish-white	Blue
229	Yellow-green	Yellow-green*	Yellow-green*	Yellow-green
183	Faint red	Red*	Red*	Red*
173	- - - - -	Red*	Red*	Red*
Solution color in daylight	Yellow	Blue	Blue	Blue

* *Intense fluorescent color.*

TABLE VIII

DESOXYCORTICOSTERONE FLUORESCENCE*

Incident light, $\tilde{\upsilon}$	Concentration of acid solution in alcohol (v/v)			
	100%	50%	25%	10%
274	Bluish-white	Rose-red	Yellow	Orange
229	Yellow-green	Yellow-green	Yellow-green	Orange-red
183	Orange	Red	Red	Red
173	Orange	Orange-red	Red	Red
Solution color in daylight	Yellow	Blue with red fl.	Blue with red fl.	Pale yellow with red fl.

* *All the colors listed are intense.*

TABLE IX

TESTOSTERONE FLUORESCENCE*

Incident light, $\tilde{\upsilon}$	Concentration of acid solution in alcohol (v/v)			
	100%	50%	25%	10%
274	Blue	Rose	Yellow-green	Yellow-green
229	Blue-green	Yellow-green	Yellow-green	Yellow-green
183	Orange-red	Red	Red	Red
173	Orange-red	Orange-red	Orange-red	Red
Solution color in daylight	Yellow	Olive-green	Green	Green

* *All colors but the orange-reds of the 100% acid solution under 183$\tilde{\upsilon}$ and 173$\tilde{\upsilon}$ incident radiation are intense.*

TABLE X *A*

PROGESTERONE FLUORESCENCE*

(Steroid dissolved in acid at 55° C.)

Incident light, $\tilde{\upsilon}$	Concentration of acid solution in alcohol (v/v)			
	100%	50%	25%	10%
274	Blue-green	Blue-green	Green	Green
229	Green-yellow	Green	Yellow-green	Orange
183	– – – – –	Red	Red	Red
173	– – – – –	Red	Red	Red
Solution color in daylight	Colorless	Faint green	Green	Colorless

* *All colors listed are intense.*

TABLE X *B*

PROGESTERONE FLUORESCENCE*

(Steroid dissolved in acid at 100° C.)

Incident light, $\tilde{\upsilon}$	Concentration of acid solution in alcohol (v/v)			
	100%	50%	25%	10%
274	– – – – –	Orange-rose	Yellow-green	Green
229	– – – – –	Yellow	Yellow-green	Yellow
183	– – – – –	Brick-red	Red	Red

* *All colors listed are intense.*

Acknowledgments

The authors wish to thank P. A. Macdonald, Director of the Manitoba Cancer Institute, for indicating the use of radiation measurements in the development of methods of hormone assay, and for the development of some of the optical instruments used in these researches.

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CULTIVATION OF POLIOMYELITIS VIRUS IN TISSUE CULTURE

I. GROWTH OF THE LANSING STRAIN IN HUMAN EMBRYONIC TISSUES[1]

By Joan C. Thicke, Darline Duncan, William Wood, A. E. Franklin, and A. J. Rhodes

Abstract

This paper presents observations on the growth of Lansing poliomyelitis virus in fluid cultures of various human embryonic and adult tissues. The evidence suggests that viral multiplication has occurred in cultures of monkey testis, human embryonic kidney, and mixtures of brain and cord. Satisfactory virus growth has been obtained particularly in cultures containing human embryonic brain and cord. Virus is present in tissue culture fluids in which the original inoculum has been diluted $10^{-23.5}$ by subcultivation. Preliminary observations suggest that a synthetic medium (Mixture 199) devised by Morgan, Morton, and Parker is superior to Hanks–Simms solution as a nutritive medium in such cultures. The cytopathogenic effect of the virus, as revealed by pH determinations and cell morphology, has been noted, although a characteristic pH differential between virus infected and control flasks was not commonly observed. Attempts to grow the virus on a larger scale in Kolle flasks are described.

Introduction

Interest has been shown for many years in the possibility that poliomyelitis virus may proliferate in tissue culture. Little experimental evidence was advanced until 1949 when Enders, Weller, and Robbins (2) reported the multiplication of the Lansing strain in cultures of the suspended cell or Maitland type employing human embryonic skin and muscle, intestine, and brain in a simple nutritive medium. These workers later showed that the Lansing strain also multiplied in human foreskin (17). Evidence has also been presented that the immunologically distinct Brunhilde strain multiplies in human embryonic skin and muscle as well as brain. Attention has also been drawn to the fact that virus accelerates the usual process of death of cells in suspended cell cultures, and the term "cytopathogenic" has been used to describe this action of virus on infected cells (11). All three types of poliomyelitis virus exert a cytopathogenic effect in cultures prepared in flasks or roller tubes, and this fact can be used in the isolation, titration, and typing of poliomyelitis viruses (12).

Confirmation of the claim that fully differentiated non-neural tissues support the growth of poliomyelitis virus was provided by Smith, Chambers, and Evans who showed that the Lansing and Hof. strains multiplied in flask cultures of adult human testicular tissue (13, 14). Tonsillar and foreskin tissues appeared to support viral growth to a limited extent, but human amnion and Wharton's jelly did not. Syverton, Scherer, and Butorac demonstrated proliferation of the Yale-SK and Lansing virus in flask cultures of testicular tissue from rhesus and cynomolgus monkeys, as well as from humans (15).

[1] *Manuscript received January 23, 1952.*

Contribution from Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont. and the Virus Research Department, Hospital for Sick Children, Toronto. This work was aided by a grant from the National Foundation for Infantile Paralysis.

Milzer *et al.* employed skin, intestine, arm and leg muscles, and brain from human embryos, and human placental tissues as a source of cells in flask cultures; they claimed to have demonstrated propagation of the Lansing strain in the human embryonic tissues, and in 2 of 13 placentas (5).

Several human embryonic and other tissues have been studied in our laboratories in a search for the most readily available tissue supporting viral growth. The results to be presented afford confirmation of the work of Enders and his colleagues, and indicate a direction in which further advances may be made.

Methods

Collection of Specimens

Human embryos of two and one-half to five months gestation were obtained from the gynaecological department of the Toronto General Hospital. They were placed in a sterile container and promptly transported to the virus laboratory of the adjacent Hospital for Sick Children. No macerated specimens were used, and in many of the embryos the heart was still beating at the time of receipt in the virus laboratory. The placental tissue used in one experiment was also obtained from the Toronto General Hospital. Human adult thyroid tissue was obtained at operation from a case of toxic adenoma, small portions of tissue being transported to the laboratory in Hanks–Simms solution containing penicillin (100 units per ml.) and streptomycin (100 μgm. per ml.).

Monkey testes were obtained from rhesus monkeys housed in the Connaught Medical Research Laboratories, located close to the Hospital for Sick Children. The organs were placed directly in Hanks–Simms solution containing antibiotics.

Nutritive Media

The nutritive fluid employed was a mixture of three parts Hanks' balanced salt solution and one part Simms' ox serum ultrafiltrate, as described by Enders *et al.* (2, 17).

A synthetic medium of chemically-defined composition known as Mixture 199, devised by Morgan, Morton, and Parker (6, 7), has also been used in certain experiments. This mixture consists of amino acids, vitamins, purines, pyrimidines, ribose, desoxyribose, a source of fatty acid, certain intermediary metabolites and accessory growth factors, and a balanced salt mixture (Tyrode's solution) that contains glucose. It has many advantages over serum ultrafiltrate, the composition of which probably varies from batch to batch. It must be realized however that Mixture 199 was developed in experiments in which only minute fragments of tissue were used. The composition is not necessarily optimal for experiments in which a substantial amount of tissue is added.

Treatment of Glassware

Glassware used in all stages of the tissue culture technique was treated with concentrated sulphuric acid containing 0.5% sodium nitrate and 0.5%

sodium chlorate. This treatment was followed by several rinses in tap water, then distilled water, and finally in glass-redistilled water before sterilization. Gray, virgin rubber stoppers* were used in preference to black rubber stoppers which have been shown to be toxic to tissues cultivated in synthetic media (8).

Lansing Virus

The strain of Lansing virus was obtained from Dr. Howard Howe of Baltimore and represented a mouse passage level greater than 200 since the original isolation of the strain by Armstrong. We have used mouse pool No. 5 prepared from the brains and cords of paralyzed mice. The LD_{50} titer of this pool, on the basis of numerous titrations in mice, has been calculated as $10^{-3.5}$. The methods used in our laboratory in the titration of Lansing virus have been previously described (10).

Preparation of Cultures

The modified Maitland technique that was used followed fairly closely the procedures employed by Weller and Enders in the cultivation of mumps virus (15). The selected tissues were first washed two or three times in a bath of Hanks–Simms solution, containing 100 units of penicillin and 100 μgm. of streptomycin per ml. Each tissue was then suspended in a small amount of this solution and minced with scissors to form pieces about 1 mm. in diameter. The minced tissue was given several further washings in Hanks–Simms solution, the excess fluid was pipetted off, and two to three drops of concentrated tissue suspension were pipetted into each of eight 25 ml. Erlenmeyer flasks which contained 3.0 ml. of Hanks–Simms solution (with 100 units penicillin and 100 μgm. streptomycin per ml.). The flasks were tightly stoppered and incubated at 36° C.

The following day the fluid elements of the cultures were removed and replaced with 3.0 ml. of fresh Hanks–Simms solution containing half the above quantities of antibiotics. To four flasks, 0.1 ml. of a 10% suspension of Lansing virus mouse pool was added, the remaining flasks serving as uninoculated controls.

In some experiments, an additional flask containing 3.0 ml. of Hanks–Simms solution with virus but without tissue was included. This flask served as a control for the possible survival of inoculated virus through the various fluid changes. All flasks were incubated at 36° C.

pH Readings

Standard solutions with the following pH values were prepared from $M/15$ Na_2HPO_4, $M/15$ KH_2PO_4, and 0.002% phenol red: 6.8, 7.0, 7.2, 7.4, 7.6, and 7.8. Three milliliters of each solution were placed in 25-ml. Erlenmeyer flasks. These flasks served as standards with which to compare the incubated flasks. For accurate pH determination in special cases, a pH meter was used.

* *Supplied by The West Co., Phoenixville, Pa.*

Maintenance of Cultures

The rate of metabolism was noted to be more rapid in the early days of a culture and appeared also to vary from tissue to tissue. The fluid elements of the cultures were changed when the pH fell to 6.8 or after three to five days. The supernatant fluids were removed as completely as possible, leaving not more than 0.2 ml. of residual fluid in the flask. Three milliliters of fresh Hanks–Simms solution (50 units penicillin and 50 μgm. streptomycin per ml.) were then added. In cultures containing synthetic Mixture 199, the pH did not drop to 6.8 owing to its stronger buffering action, and fluids were therefore changed arbitrarily every four days.

The fluids from the virus-infected flasks were pooled, centrifuged at 2000 r.p.m. for 10 min., and the supernatants stored frozen. The control flasks were treated likewise.

Fluids were changed as described, until the cells had been incubated for about 28 days. Usually about 8 to 10 changes of fluid were made during this period. In Mixture 199, cell metabolism was observed to continue for over 50 days.

Subcultivation

When Hanks–Simms medium was used, changes in pH were no longer observed after 16-28 days. It is inferred that the cells were no longer metabolizing. A subculture was then initiated, using tissue consisting of cells of similar type, freshly obtained. The flasks were incubated for one day and then inoculated with 0.1 ml. of fluid from one of the later fluid changes of the previous culture. Usually four flasks were inoculated with fluid supposedly containing virus, and four flasks with fluid from the control series. All flasks were then incubated and fluid changes made every few days as above described, to continue the subculture.

Infectivity Tests

(*a*) *Mice*

The mice used were from 10-16 gm. in weight and were obtained from the Connaught Medical Research Laboratories or Tumblebrook Farms, N.Y.

Groups of 6 to 10 mice were injected cerebrally (0.03 ml.) with each pool of fluid withdrawn from supposedly infected cultures. In addition, portions of the cells remaining at the conclusion of the culture were ground and the suspension inoculated in mice. These mice were carefully examined for paralysis and many were also examined histologically. The clinical and histological pictures were typical of Lansing infection in the mouse.

On several occasions, pooled fluid from the control flasks of a culture as well as suspensions of pooled cells from such flasks were inoculated into mice, and only very occasionally did death occur. These mice did not show paralysis. This procedure served as a check that no mouse pathogenic agent derived from human or monkey tissue was being propagated.

On certain occasions, serial 10-fold dilutions of fluid were inoculated into groups of 20 mice to titrate the virus content. LD_{50} end points were

calculated by the Kärber method (3) which gives results very similar to those obtained by the probit or Reed and Muench method (9).

A neutralization test with Lansing immune monkey serum was carried out on fluid changes from several cultures. Mixtures of serum and infected fluids were allowed to stand for one hour at room temperature and inoculated in groups of 20 mice along with suitable controls to test for neutralization.

(*b*) *Monkeys*

Several transfer fluids were inoculated thalamically in rhesus monkeys (0.8 ml.); in some cases fluids were diluted 1 : 10 or 1 : 100. All animals were killed for histological examination after four – six weeks, or at the onset of paralysis.

Concentration of Fluids by Ultracentrifugation

An attempt was made to concentrate the virus present in pooled infected tissue culture fluids. The fluids were centrifuged at 40,000 r.p.m. in a preparative "Spinco" ultracentrifuge. The deposits from the 12 tubes were taken up in about 1 ml. of supernatant fluid and stored frozen. As the total volume of fluid centrifuged was 130 ml., and as the final volume was 1.0 ml., a theoretical concentration of 130-fold was achieved.

Attempts to Grow Virus in Larger Flasks

Preliminary experiments were made on the growth of poliomyelitis virus in Kolle flasks. In order to keep the depth of nutrient medium and the proportion of tissue to fluid the same as before, all constituents were added in quantities approximately 11 times greater.

Histological Examination of Tissues

At the termination of each culture, portions of the residual tissue fragments from the various flasks were pooled, fixed either in Zenker's fluid or formalin, sectioned, and stained with haematoxylin and eosin. The control tissues were treated in a similar manner.

Results

The following series of experiments were performed:

(1) Preliminary experiments in which original cultures of a number of human embryo tissues together with human adult thyroid and placenta were carried.

(2) The virus growth-supporting properties of monkey testis and human embryonic lung were examined more intensively, and virus subcultures were made.

(3) Second and fourth subcultures of virus were made in human embryonic kidney and mixtures of brain and cord respectively.

(1) *Preliminary Experiments*

The results of a number of preliminary experiments in which several human embryonic tissues and adult thyroid and placenta were used are shown in Table I. These experiments were performed as a general introduction to the technique, and in an attempt to reproduce the results of Enders.

TABLE I

CULTIVATION OF LANSING VIRUS IN HUMAN CELLS

Designation of tissue	Amount of virus added to cultures	Deaths in groups of mice inoculated with the following transfer fluids								Deaths in mice inoculated with pooled cells	Duration of culture (days)
		1	2	3	4	5	6	7	8		
Human embryonic tissues											
Kidney		6/6*	6/6	6/6	6/6	6/6	6/6	4/6	4/6†	5/5	29
Gut (1)		5/5	3/6	6/6	3/6	6/6	4/5	5/6		6/6	29
Gut (2)		5/5	4/4	5/6	2/5	0/6	0/6	1/6		2/6	31
Thymus (1)		5/6	6/6	6/6	6/6	5/6	1/6	0/6		5/6	29
Thymus (2)	1000 LD_{50}	3/6	6/6	5/6	5/5	2/6	0/6	0/6		3/6	31
Heart		6/6	6/6	6/6	5/6	0/6	0/5	0/6		0/6	30
Spleen		6/6	5/5	5/5	5/6	0/6	0/6	1/6		0/6	31
Liver		5/6	4/6	2/6	1/6	0/6	1/6	0/6		0/6	30
Adrenal		6/6	6/6	2/6	1/6	0/6	0/6	0/6		1/5	30
Skins and muscle (1)	100 LD_{50}	1/5	3/6	4/5	5/6‡	2/6	0/6	0/6		2/5	29
Skin and muscle (2)	1000 LD_{50}	—	5/6	5/6	3/6	3/5	0/6	0/6		0/6	29
Skin and muscle (3)		6/6	6/6	6/6	0/6	0/6	0/6	0/5		0/6	31
Human adult tissues											
Placenta (spongiose tissue)	100 LD_{50}	2/6	3/6	0/6	3/6	0/6	0/6	0/5		4/5	29
Thyroid	1000 LD_{50}	8/8	6/6	2/6	0/6	0/6	1/6	1/6	1/6	6/6	32

** The denominator of each fraction represents the number of mice injected, and the numerator the mice dying within a four-week period.*

† Two rhesus monkeys inoculated thalamically with 0.8 ml. of this fluid undiluted and diluted 1 : 10 became paralyzed and showed histological changes typical of Lansing poliomyelitis.

‡ A rhesus monkey inoculated thalamically with 0.8 ml. of this fluid became paralyzed and showed histological changes typical of Lansing poliomyelitis.

It is probable that some of the original virus inoculum was carried over by simple dilution, and that the third fluid change accordingly contained enough virus to infect mice. However, in this series of experiments, deaths occurred in later fluid changes of cultures of several organs, and some virus growth may therefore have occurred. It is probable however that there was definite virus growth only in cultures of human embryonic kidney and gut. It will be noted that virus was recovered from the suspensions of the pooled cells remaining at the end of the culture even though no virus appeared to be present in later fluid changes.

(2) *Growth in Monkey Testis and Human Lung*

In the experiments recorded in Table II attempts were made to propagate virus in subculture. Fluids from the later transfers of the original cultures

TABLE II

SUBCULTIVATION OF LANSING VIRUS IN MONKEY TESTIS AND HUMAN EMBRYO LUNG

Designation of tissue	Designation of culture	Source and amount of virus added to cultures	Deaths in groups of mice inoculated with following transfer fluids								Deaths in mice inoculated with pooled cells	Duration of culture (days)
			1	2	3	4	5	6	7	8		
Monkey testis	Original culture	1000 LD_{50}	7/7	1/6	4/8	8/8	4/5	6/6*			4/4	24
	1st subculture	0.1 ml. of 6th fluid change of original culture	3/6	2/6	4/6	6/6	6/6	6/6	5/6*		6/6	29
Human embryonic lung	Original culture	1000 LD_{50}	5/6	6/6	6/6	6/6	6/6*	4/6	0/6		6/6	30
	1st subculture	0.1 ml. of 5th fluid change of original culture	0/6	0/6	0/6	0/6	3/6	5/6*	5/6	0/6	0/6	29

* *Rhesus monkeys inoculated thalamically with 0.8 ml. of these fluids became paralyzed and showed histological changes typical of Lansing poliomyelitis.*

were subinoculated into freshly prepared cultures. The tissues used were human embryonic lung and monkey testis. In monkey testis the occurrence of deaths in mice inoculated with all seven transfer fluids is very suggestive of viral multiplication. In the experiment with human embryonic lung, deaths occurred only in mice inoculated with transfer fluids 5, 6, and 7, as if the virus required several days in which to multiply to a level sufficient to infect mice.

It may be assumed that the original inoculum of 0.1 ml. of virus suspension is diluted 1 in 30 when added to the 3 ml. of nutritive fluid at the initiation of each culture, and that each time the fluid is withdrawn and replaced a further dilution of 1 : 15 occurs. On the basis of this calculation, the original virus inoculum was diluted $10^{-15.9}$ in the seventh fluid change of the first subculture of monkey testis. At the seventh fluid change of the first subculture of human embryonic lung the dilution was $10^{-14.7}$. It would appear therefore that viral proliferation definitely occurred in these organs.

(3) *Growth in Brain and Cord and in Kidney*

The results of two series of cultures of human embryonic kidney and of brain and cord in which the virus was carried to a second and fourth subculture

TABLE III

SUBCULTIVATION OF LANSING VIRUS IN HUMAN EMBRYONIC KIDNEY AND BRAIN AND CORD

Designation of tissue	Designation of culture	Source and amount of virus added to cultures	Deaths in groups of mice inoculated with the following transfer fluids											Deaths in mice inoculated with pooled test cells	Duration of culture (days)
			1	2	3	4	5	6	7	8	9	10	11		
Human embryonic kidney	Original culture	0.1 ml. of 10% Lansing mouse pool (1000 LD_{50})	5/6	6/6	5/6	5/5	6/6	5/5	4/5	6/6*				2/3	31
	1st subculture	0.1 ml. of 7th fluid change of original culture	0/6	0/6	0/6	5/6*	4/6	4/6	0/6	0/6	1/6	0/6		2/3	38
	2nd subculture	0.1 ml. of 4th fluid change of 1st subculture	0/6	1/6	3/6	2/6	6/6	5/6	6/6*	6/6	4/6	3/6	5/6	4/4	31
Human embryonic brain and cord	Original culture	0.1 ml. of 10% Lansing mouse pool (1000 LD_{50})	5/5	6/6	5/6	2/2	5/6	4/5*	4/5					5/5	30
	1st subculture	0.1 ml. 6th fluid change of original culture	0/6	1/6	5/6	6/6	6/6	6/6	6/6†	5/6	5/6			5/6	31
	2nd subculture	0.1 ml. of 7th fluid change of 1st subculture	1/6	6/6	6/6	6/6	6/6*	4/6	2/6	0/6				6/6	32
	3rd subculture	0.1 ml. of 5th fluid change of 2nd subculture	0/6	6/6	5/6*	6/6	5/6*	2/6	2/6	3/6	5/6	0/6		0/5	39
	4th subculture	0.1 ml. of 4th fluid change of 3rd subculture	3/5	2/6	4/6	5/6	6/6*	3/6	3/6	1/6	0/6	1/6		2/6	36

* *Rhesus monkeys inoculated thalamically with 0.8 ml. of these fluids undiluted became paralyzed and showed histological changes typical of Lansing poliomyelitis.*

† *Two rhesus monkeys inoculated thalamically with 0.8 ml. of this fluid diluted 1 : 10 and 1 : 100 became paralyzed and showed histological changes typical of Lansing poliomyelitis.*

respectively are recorded in Table III. There seems to be no doubt that virus proliferated, as mice and monkeys were killed with later transfer fluids of each subculture. In the kidney series, the dilution of the original virus in the 11th transfer fluid of the second subculture has been calculated to be $10^{-26.7}$. In the brain series, the dilution of the original inoculum of virus by the time the fourth transfer fluid of the third subculture was reached is $10^{-27.1}$. The dilution of the original inoculum in the fifth transfer of the fourth subculture is $10^{-33.3}$. The development of typical Lansing poliomyelitis in monkeys inoculated with later transfer fluids is of particular interest.

Titration of Transfer Fluids

The transfer fluids of several cultures were titrated in mice. A representative example of such a titration is shown in Table IV. The highest titers of virus were usually found in transfer fluids 3 through 6, the amount of virus decreasing in later fluids.

TABLE IV

TITRATION OF TRANSFER FLUIDS FROM AN ORIGINAL CULTURE OF VIRUS IN HUMAN EMBRYONIC KIDNEY*

Human embryonic kidney (Original culture)		Human embryonic kidney (Original culture)	
Fluid change	LD_{50} titer in mice	Fluid change	LD_{50} titer in mice
1	$10^{-1.97}$	5	$10^{-1.92}$
2	$10^{-1.65}$	6	$10^{-1.16}$
3	$10^{-2.10}$	7	$10^{-0.95}$
4	$10^{-1.55}$	8	$10^{-0.25}$

* *All titrations carried out by inoculating log dilutions of fluids in groups of 20 mice.*

pH Changes

It was reported by Robbins, Enders, and Weller (11) that growth of poliomyelitis virus in tissue culture impairs cellular metabolism, as manifested by decreased acid production by the virus infected tissues as compared with the control tissues. Immediately prior to a transfer of fluid the average pH of the control flasks was said to be 0.2 units or more lower than that of the virus infected flasks. This phenomenon was noted in two of our experiments employing human embryonic brain and cord, and Fig. 1 illustrates the average pH readings of test and control flasks in an original culture. On the 23rd day of culture there was a difference of 0.55 pH units between test and control flasks. In other experiments no such differences were observed.

Synthetic Mixture 199

An experiment was carried out to compare virus production in cultures containing Mixture 199 and in others containing the customary Hanks–Simms solution. Human embryonic brain and cord from the same embryo was

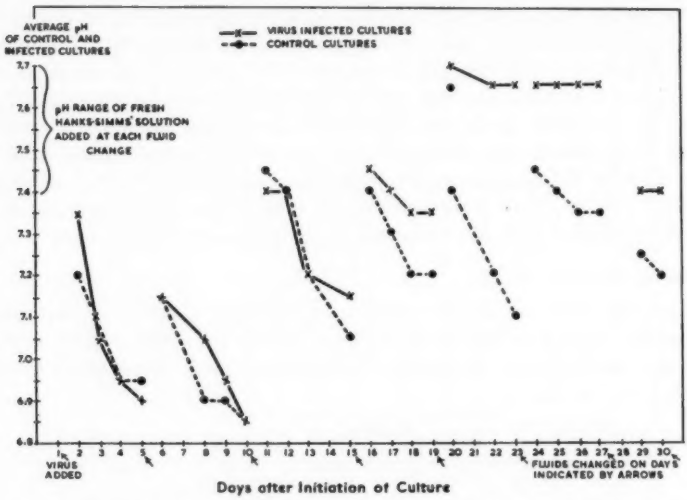

FIG. 1. Changes in pH in virus infected and control flasks of an original culture using human embryonic brain and cord.

employed in each series of flasks. The results of inoculating various transfer fluids into mice are shown in Table V. It will be seen that virus could be demonstrated in all transfer fluids 1 through 16 in Mixture 199, whereas there was virtually no evidence of the presence of virus in fluids 10 through 15 of the Hanks–Simms medium series.

Identity of Virus

The identity of the cultivated agent with Lansing virus was confirmed by the finding of typical histological changes in the central nervous system of paralyzed mice and monkeys, and by neutralization tests with certain fluid changes. Thus the virus present in the sixth fluid change of the original culture of virus in monkey testis was completely neutralized by known Lansing immune serum prepared in monkeys and not by control normal monkey serum. A similar result was obtained with the fourth fluid change of the third subculture of virus in brain and cord.

Survival in Absence of Cells

On a number of occasions, virus was inoculated into 3 ml. of Hanks–Simms medium without cells. The flasks were incubated along with test flasks, and transfers were also performed. Virus could be detected in the first fluid change but only exceptionally in the second.

Concentration of Virus from Tissue Culture Fluids

A pool of virus-infected culture fluids was concentrated as already described. A portion of the pool before centrifugation and a portion of the resuspended

TABLE V

COMPARISON OF VIRUS PRODUCTION IN HUMAN EMBRYONIC BRAIN AND CORD CULTIVATED IN HANKS–SIMMS MEDIUM AND IN SYNTHETIC MIXTURE 199*

Inoculum	Deaths in groups of mice	
	Hanks–Simms	Mixture 199
Test fluid change		
1	5/6	6/6
2	2/6	5/6
3	6/6	6/6
4	5/6	6/6
5	3/6**	5/6**
6	4/6	5/6
7	3/6	2/6
8	1/6	6/6
9	1/6	5/6
10	0/6	6/6
11	1/6***	6/6**
12	0/6	4/6
13	0/6	3/6
14	0/6	5/6
15	0/6***	6/6**
16	—	3/6
17	—	0/6
18	—	0/6
19	—	0/6
Pooled test cells	0/6***	6/6**
Total deaths	31/96	86/120

* *Cultures inoculated with 500 LD_{50} of Lansing virus, mouse pool.*

** *Monkeys inoculated thalamically with 0.8 ml. of fluid developed poliomyelitis, typical clinically and histologically.*

*** *Monkeys inoculated thalamically showed no clinical or histological evidence of poliomyelitis.*

high speed deposit were titrated by inoculation of 10-fold dilutions in groups of 20 mice. The LD_{50} titer of the original pool was $10^{-1.25}$ and that of the resuspended deposit $10^{-2.8}$. The actual concentration of infectious particles thus appears to have been about 35-fold.

Attempts to Grow Virus in Larger Flasks

Table VI shows the results of a culture of virus in monkey testicular tissue inoculated with mouse pool, and held in Kolle flasks. Three test and three control flasks were set up and each fluid change was inoculated undiluted and diluted 1 : 10 into groups of 10 mice.

As deaths occurred in mice inoculated with the sixth transfer fluid in a 1 : 10 dilution, it seems probable that viral proliferation occurred in these larger containers.

Histology of Tissue Fragments

The tissues were stained and sectioned as previously described. To the naked eye, the control tissues appeared to have taken up more haematoxylin

TABLE VI

EXPERIMENT WITH MONKEY TESTICULAR TISSUE IN KOLLE FLASKS

Amount of virus added to cultures	Dilution of fluid change	Deaths in groups of mice inoculated with following transfer fluids										Deaths in groups of mice inoculated with pooled test cells	Duration of culture (days)
		1	2	3	4	5	6	7	8	9	10		
1.2 ml. of 10% suspension of Lansing mouse pool	Undiluted	10/10	7/10	9/10	10/10	8/10	2/10	1/10	0/10	0/10	0/9	1/9	39
	Diluted 1 : 10	9/10	3/10	5/10	9/9	6/10	3/10	0/8	0/10	0/10	0/9		

than their virus-infected counterparts and stained a deeper blue. On microscopic examination, both control and virus-infected tissues showed much cellular necrosis with nuclear fragmentation and pycnosis, although the virus-infected tissues appeared to be more severely affected than the controls.

Discussion

The results recorded in this paper indicate that Lansing poliomyelitis virus multiplies in tissue cultures prepared with various human cells suspended in Hanks–Simms medium. In this regard, the work affords confirmation for the claims of Enders and his colleagues.

One of our interests in this field lies in investigating the possibilities of preparing large quantities of virus suitable for use as a vaccine. It is therefore first necessary to study in some detail the basic conditions in which poliomyelitis virus proliferates *in vitro*, in order that a reproducible technique for the preparation of virus can be developed. In particular it is desired to obtain virus from media containing only human antigenic material.

In preliminary experiments directed towards these objectives, we obtained suggestive evidence that Lansing virus multiplies in a variety of human embryonic tissues such as kidney, brain and cord, gut, thyroid, liver, and thymus. In more extensive experiments, involving subcultivation and therefore a more considerable dilution of the original virus inoculum, definite evidence of viral growth was obtained in monkey testis and human embryonic lung, kidney, and mixtures of brain and cord. More detailed studies were made of two human embryonic tissues of different type, brain and cord, and kidney. In the brain and cord series, evidence of viral growth was obtained in the fifth transfer fluid of the fourth subculture. Virus multiplied successively therefore in association with cells derived from five different human embryos. It has been calculated that the original virus inoculum of 0.1 ml. of 10% mouse brain suspension was diluted $10^{-33.3}$ by the time the fifth fluid change of the fourth subculture was reached. The identity of the agent propagated was proved by inoculation of monkeys, and the performance of a virus neutralization test with immune serum prepared in monkeys. The agent present in the transfer fluids of the fourth subculture of brain and cord induced typical Lansing poliomyelitis in monkeys and mice, and was neutralized by immune but not by normal monkey serum. It would appear therefore that it should be possible to propagate Lansing virus in a variety of human embryonic tissues.

It should be noted that in one regard our results differ somewhat from those published by Enders and his colleagues, for we have not commonly demonstrated a difference in pH between virus infected and control tissue cultures in Hanks–Simms medium. The reason for this is not at present apparent, but it may be noted that other workers have likewise found minimal pH changes in cultures of Lansing virus (4).

In most of the experiments recorded in this paper, we used the simple medium recommended by Enders consisting of Hanks' balanced salt solution

with Simms' bovine serum ultrafiltrate. In the later stages of our work, and in studies now actively in progress, we have used a more elaborate synthetic medium devised by our colleagues Morgan, Morton, and Parker. Although Mixture 199 is still incomplete in that it will not support cell life indefinitely, it is currently being studied by Parker and his colleagues as a basal medium for the investigation of additional nutritional substances.

Mixture 199 has certain theoretical advantages over Hanks–Simms medium. For example, it is of defined chemical composition, and different batches should not vary appreciably one from another; in particular, Mixture 199 does not contain serum ultrafiltrate which is of undefined composition. Mixture 199 contains a much more efficient buffering system than does Hanks–Simms medium, and the pH does not therefore fall as rapidly. Using Hanks–Simms medium, we have found that with certain tissues, in the early stages of a culture, it is necessary to remove and replace the fluid elements every 48 hr. This rapid replacement of nutritive material may not allow of virus growth. Further, the composition of Mixture 199 is such that the tissue cells should survive for very much longer periods than in Hanks–Simms medium, even though the medium was devised by means of techniques involving very small cell populations.

In practice, these various theoretical advantages of the synthetic medium were realized. In particular, because of the buffering action, replacements of fluid in the early stages of the cultures were made less frequently than were necessary with cultures containing Hanks–Simms medium. It was also found that cell metabolism, as evidenced by alterations in pH, continued for over 50 days in Mixture 199, whereas after 21-28 days in Hanks–Simms medium, the cellular components of the cultures appeared to have ceased to metabolize, as judged by pH readings.

Associated with this prolongation of the survival period of the cells was the finding that production of virus continued over a longer period of time. With Hanks–Simms medium, transfer fluids 1 through 9 contained virus as judged by the inoculation of mice, but no virus could be detected in six subsequent transfer fluids. By contrast, with Mixture 199, virus was present in transfer fluids 1 through 16. The use of Mixture 199 should facilitate studies of the metabolic requirements of Lansing virus such as those reported by Brown and Ackermann (1).

Our investigation of the basic biological conditions necessary for the optimum yield of Lansing virus in tissue cultures are proceeding, and various modifications of synthetic Mixture 199 are being made in our laboratory in an effort to meet the special requirements of large masses of tissue.

Acknowledgments

The authors are grateful to the members of the obstetrical, gynaecological, and surgical staff of the Toronto General Hospital who have kindly made available tissue for this study. Dr. Raymond C. Parker, Dr. Joseph F. Morgan, and Miss Helen J. Morton, of the Connaught Medical Research

Laboratories, have extended to us much helpful advice and practical assistance, and have furnished us with supplies of their synthetic media.

The authors owe a great debt to Dr. John Enders and his colleagues for much help and advice extended during the past 18 months. This help has included the personal demonstration to some of us of their tissue culture methods.

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A RAPID ULTRAFILTRATION APPARATUS[1]

By I. W. Coleman[2]

Abstract

An apparatus for the preparation of protein-free filtrates of biological fluids for subsequent amino-acid analysis by paper chromatography is described. The construction allows filtration to be carried out at high pressures, hence increasing the rate of filtration. Contamination by interfering heavy metal ions is avoided by a design in which solutions are exposed only to lucite.

Introduction

In the application of paper chromatography to the analysis of biological solutions for amino acids, the prior removal of proteins and peptides of high molecular weight is necessary since these hamper resolution (1). The customary procedures of heavy metal and cationic precipitations are of limited use under these conditions, since they introduce higher salt concentrations which are deleterious to the chromatographic process and may involve the loss of some of the amino acids under investigation. Although alcohol and acetone precipitation of protein have been used by the author with favorable results, the procedure of ultrafiltration recommended by Dent and Schilling (1) has been found simpler and more reliable. However, the technique of Greenberg and Gunther (2) adopted by these workers has a number of serious shortcomings. The collodion membranes prepared for this procedure are delicate and since they are used unsupported, the filtration pressures used cannot greatly exceed 150 mm. Hg. This results in a very low filtration rate. Nicholas (4) avoided these difficulties by replacing the collodion with cellophane, and supported the cellophane membrane with a silver screen such that pressures as high as 200 p.s.i. could be applied. However, his apparatus is not applicable to ultrafiltration of solutions for chromatography. A copy of his apparatus constructed in this laboratory was found to introduce copper ions into the ultrafiltrates in sufficient quantity to give a positive test with diethyldithiocarbamate. It was suspected that the brass construction led to the contamination, since heavy plating of the copper screen used with nickel did not eliminate the copper contamination. It is of prime importance to reduce heavy metal contamination of the ultrafiltrates before chromatography, since these interfere with the ninhydrin reaction used to locate the amino acids (3). This difficulty has been circumvented by constructing an apparatus in which all parts exposed to the solutions are of chemically inert materials. But for the difficulty of working, glass would be the ideal material for such a purpose. Several all glass designs were considered, but were abandoned in favor of the use of lucite. This material is easy to work and can be machined to close

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Contribution from the Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg, Man.

[2] *Present address: Department of Pathology, St. Boniface Hospital, St. Boniface, Man.*

tolerance with the customary machine shop equipment. In the design finally adopted the only metal with which the solutions come in contact is the stainless steel mesh used to support the membrane. This does not introduce metallic contaminants in sufficient concentration to interfere with any subsequent procedures.

The Apparatus

The apparatus is shown in section in the exploded diagram, Fig. 1. It consists of a lucite filtering chamber 3.0 cm. inside diameter, having a capacity of 75 ml. This is supported by a brass jacket fitted with a filling hole and a

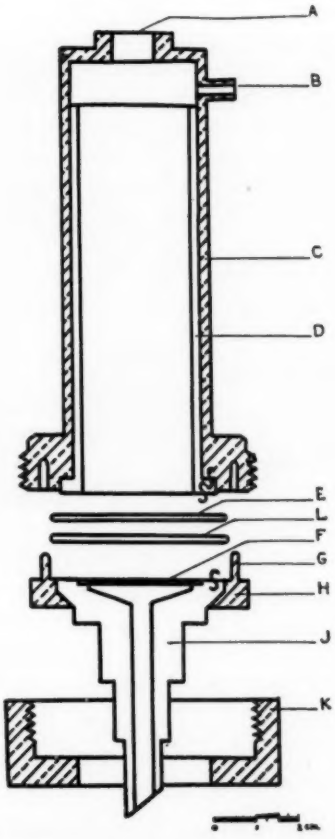

FIG. 1. Exploded section diagram of ultrafiltration apparatus: A, filling hole threaded for 3/8 in. plug; B, connection to valve and pressure manifold; C, brass tube; D, lucite chamber; E, cellophane dialyzing membrane; L, filter paper; F, stainless steel screen; G, centering pins; H, brass support ring; J, lucite collecting funnel; K, brass clamping collar.

shutoff valve connecting the chamber to a pressure manifold. The cellophane membrane supported by an acid-washed filter paper is clamped in close contact with the flange of the filtering chamber by the lucite collecting funnel fitted with a 40 mesh stainless steel screen. Tight contact is achieved by a threaded brass collar. One feature of the apparatus is the use of the centering pins which allow the collar to be turned tightly without any danger of tearing the membrane. No gaskets are required for pressures as high as 100 p.s.i., provided the plastic flanges have been machined smooth and flat. Pressure is applied directly on the solutions, using compressed air or nitrogen tanks controlled by the usual diaphragm valve. For most work, the pressure applied need not exceed 60 p.s.i. A multiple unit constructed and in constant operation in the author's laboratory is shown in Fig. 2.

The ease with which the apparatus can be taken down and reassembled allows thorough cleaning. Care must be taken to avoid the use of organic solvents, e.g., toluene, as preservatives of solutions undergoing filtration. Such solvents soften the plastic and tend to etch the machined surfaces of the flanges so that a pressure seal cannot be made. The use of a small amount of allylisothiocyanate to inhibit bacterial and mold growth in samples is recommended.

TABLE I

RECOVERY OF ADDED AMINO ACIDS IN ULTRAFILTRATES

Protein solution				Ultrafiltrate			
Armour crystalline bovine plasma albumin, 2% plus	Mgm. per 100 ml.	Amino acid nitrogen calculated, mgm./ml.	Volume filtered, ml.	Fraction analyzed, ml.	Amino acid nitrogen determined, mgm./ml.	Net amino acid nitrogen	Per cent recovery
Glycine	0	0	20	First 5.0	0.012	—	—
	100	0.187	20	First 5.0	0.201	0.189	100.1
	400	0.749	20	First 5.0	0.747	0.735	98.2
	560	1.39	20	First 5.0	1.39	1.38	99.5
Glycine	250	0.467	20	First 2.0	0.472	0.459	98.6
				2nd 2.0	0.474	0.462	99.3
				5th 2.0	.476	0.464	99.8

Under a constant pressure of 60 p.s.i., aqueous solutions of plasma albumin, lactalbumin, casein, and egg albumin can be quantitatively cleared of protein at filtration rates of 1.5–2.0 ml. solutions cleared per hour. The ultrafiltrates from each of these solutions showed no evidence of precipitate when tested with trichloroacetic, tungstic acid, or phosphotungstic acid. As well, no

PLATE I

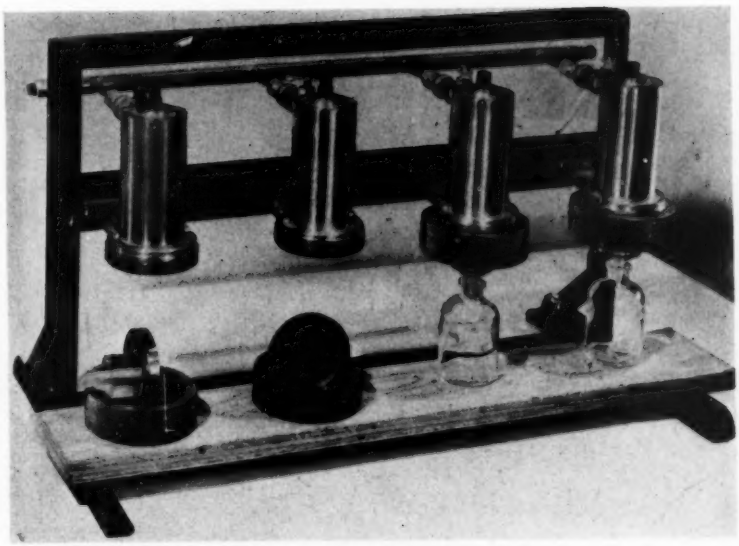

FIG. 2. Photograph of apparatus as constructed.

evidence for the presence of protein could be obtained from the biuret, xanthoproteic, or Hopkins-Cole reactions. These rates were measured at protein concentrations of 2.0 gm. per 100 ml. using a single layer of cellophane dialyzing tube as the membrane.

To test the recovery of amino acids in the filtrate, bovine plasma albumin (Armour) solution 2% (w/v) was prepared. Known quantitites of glycine were added to this solution, of which amino acid nitrogen in the ultrafiltrates as determined by the method of Pope and Stevens (5) indicated a recovery of 98 to 100% of the glycine added. No significant variation in this recovery was noted when successive fractions of the ultrafiltrate were examined (See Table I).

With blood serum or plasma the rate is somewhat slower, from 1.2–1.8 ml. per hour, depending on the protein content. Since small volumes of solution are required in partition chromatography, these rates allow three or four samples to be cleared of protein in the average working day.

Acknowledgment

The author is indebted to Mr. W. R. Jones for his help in the construction of the apparatus.

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TULAREMIA AMONG BEAVER AND MUSKRAT IN ONTARIO[1]

By N. A. Labzoffsky[2] and J. A. F. Sprent[3]

Abstract

During a 1949-51 epizootic of unknown etiology in Northern Ontario, six beavers, four beaver livers, and one muskrat were examined. Two beaver carcasses were obtained from Sioux Lookout, one from Dryden, one from Port Arthur, two from Gananoque, and four beaver livers from Sudbury. One muskrat carcass was forwarded from Long Point district. *Pasteurella tularensis* was isolated from five beavers and the muskrat, by guinea pig and developing chick embryo inoculation. This disease is apparently geographically widespread in Ontario among the animals, since the organism was isolated from the specimens obtained from such widely separate areas as Patricia, Long Point, and Gananoque districts.

Introduction

The occurrence of tularemia in beaver in the United States was first described by Hammersland and Joneschild (3) and Scott (15). Later Jellison *et al.* (5) reported an isolation of *Pasteurella tularensis* from the tissue of dead beavers from seven of the eight areas involved in an epizootic in Montana in 1939-40. Observations made over a period of years by Parker and his coworkers (13) on the epidemiology of tularemia and reported in a recent extensive communication indicate that tularemia epizootics among beaver and muskrat in the Northwestern United States are widespread. *P. tularensis* was isolated by them from dead beavers or muskrats from central and western Montana, northern Wyoming, southern Idaho, northern Utah, and the Klamath Lake region of Oregon.

Indirect evidence of the occurrence of tularemia among muskrat and beaver in Canada was obtained in Manitoba where "at least two trappers have contracted the disease from handling bodies of diseased animals" (1). Earlier Johns (8) reported three human cases of tularemia in Ontario, all associated with the skinning of trapped muskrats.

In 1949 an extensive outbreak of disease occurred among beaver in Patricia district in Ontario and the authors were asked to investigate the etiology of this epizootic. In the course of this investigation, beavers from several other districts as well have been examined, with the result that *P. tularensis* has been isolated from beavers from three widely separate areas.

Preliminary results of this investigation have been reported elsewhere (18). In the present communication the observations made during this limited survey are presented in greater detail.

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Contribution from the Department of Health of Ontario and the Ontario Research Foundation.

[2] *Virus Section, Central Laboratory, Department of Health of Ontario, Toronto, Ont.*

[3] *Department of Parasitology, Ontario Research Foundation, Toronto, Ont.*

Material and Methods

A total of six beavers, four beaver livers, and one muskrat were examined for the presence of tularensis infection. Two beaver carcasses were obtained from Sioux Lookout, one from Dryden, one from Port Arthur, two from Gananoque, and four beaver livers from the Sudbury district. One muskrat carcass was forwarded from the Long Point district.

All carcasses arrived at the laboratory in a good condition and were either autopsied immediately or kept frozen until the time to do so was available.

Using aseptic technique spleen and liver from each animal were removed; a portion of each of these organs was reserved for inoculation and for direct microscopic examination. The remainder was stored in a carbon dioxide cabinet for future reference. A pooled organ suspension from each animal was made in saline and inoculated into six guinea pigs, three of which usually received the inoculum subcutaneously and three intraperitoneally in 0.5 – 1 cc. amounts.

If, on direct microscopic examination, the inoculum was found to be free of contaminants, the material from each beaver was also inoculated into 12 eight-day-old developing chick embryos, using yolk sac inoculation technique. The dose in this case varied from 0.1 cc. to 0.5 cc.

Inoculated guinea pigs were observed daily and were sacrificed when the signs of illness were obvious. Spleens from these animals were repassaged either in guinea pigs or developing chick embryos. Surviving guinea pigs were kept under observation up to six weeks, when they were sacrificed after a specimen of blood was obtained. Various organs from these animals were cultured on cystine-blood-agar and sera were tested for the presence of agglutinins for *P. tularensis*.

The inoculated eggs, which were incubated at 37° C., were, likewise, observed daily. Embryos dying 48 hr. after inoculation and later were examined for the presence of *P. tularensis*. All surviving eggs were opened as a rule on the seventh day after inoculation.

Cystine-blood-agar medium was used for *in vitro* cultivation of the isolated organism. The isolated cultures were verified as *P. tularensis* by an agglutination technique using known *P. tularensis* antiserum.

Pathological Findings

Gross Pathology

Beaver

The pathological changes referred to here were observed in five beavers from which *P. tularensis* was isolated and in one beaver from which no organism was recovered. Skins of the beavers delivered to the laboratory unpelted were free from abrasions; all carcasses were fat and did not show any signs of prolonged illness. Subcutaneous tissues as a rule were injected and several patches of hemorrhagic infiltration were present. Enlarged and hemorrhagic superficial lymph nodes were observed in some cases but not in

others. Peritoneal and pleural cavities invariably contained copious amounts of blood-tinged exudate. No other pathological changes were observed in four specimens. In the remaining two, however, in addition to the above abnormalities, the spleen was enlarged and it, the liver, and mesenteric lymph nodes were densely studded with grayish-yellow foci of necrosis. The pericardial sac was thickened and contained bloody exudate. The kidneys were intensely congested, soft, and pulpy with well pronounced demarcation between the cortex and medulla.

Pathological changes in some of the beaver carcasses examined in the field, quantitatively speaking, were more pronounced (17).

Muskrat

No skin abrasions were present. The carcass was that of a well nourished animal.

Subcutaneous tissues were injected and patchy hemorrhagic infiltrations were present. Pleural and peritoneal cavities contained a large quantity of bloody exudate. The spleen and liver appeared normal, the kidneys were congested, dark, and somewhat enlarged; mesenteric lymph glands enlarged and hemorrhagic. The changes on the whole were those of an acute infectious process.

Guinea Pig

Guinea pigs inoculated with beaver material and succumbing to the disease were invariably emaciated. The visible mucosae were dry and somewhat cyanotic. Superficial lymph nodes, particularly inguinal, were enlarged and congested. Peritoneal, pleural, and pericardial cavities contained an abundance of hemorrhagic exudate. In animals dying on the sixth day or later after inoculation the spleen was enlarged, dark, hard, and densely studded with grayish-yellow foci of necrosis. The capsule was greatly thickened. The liver, likewise, was enlarged, congested, and contained similar foci of necrosis, but they were not so numerous. Occasionally enlargement and congestion of one or both kidneys was noted.

In guinea pigs receiving a heavier inoculum and dying earlier than the fifth day, the pathological changes were less pronounced than in those receiving a more dilute inoculum and dying at a later date.

Direct Microscopic Examination

Direct microscopic examination of impression smears of spleen and liver from the beavers (including the one from which *P. tularensis* was not isolated) and the muskrat revealed the presence of numerous tiny coccoid bodies, which were more prevalent in splenic than in hepatic tissue. These bodies stained blue with Castaneda stain and were Gram-negative. The same microscopic picture was observed in the spleen and liver of infected guinea pigs and in the yolk sac of infected chick embryos.

Isolation and Identification of the Organism

Cultural and Morphological Characteristics of the Organism

A bacterial agent was isolated, both by guinea pig and egg inoculation from hepatic and splenic tissues of one muskrat (Long Point) and from five beavers. Two beavers were from Sioux Lookout, one from Dryden, and two from Gananoque.

The beaver strains failed to grow on cystine-blood-agar on initial isolation. However, after one or two passages in guinea pigs, the *in vitro* propagation of the beaver strains, using cystine-blood-agar, presented no difficulty. It is of interest to mention that on isolation from guinea pigs, the organism grew more readily on medium containing guinea pig rather than rabbit or human blood. On the medium containing guinea pig blood, growth was associated with extensive hemolysis, whereas in the case of rabbit or human blood, the phenomenon was not observed. The muskrat strain, on the other hand, on initial isolation, grew on cystine-blood-agar but only with difficulty. In this case only scanty growth, consisting of a few colonies, was observed after four days of incubation at 37° C. Subsequent subcultures, however, were easily propagated.

The growth on the cystine-blood-agar medium of both the beaver and muskrat strains appeared as minute, grayish-white, viscous colonies. After one or two subcultures the organism appeared as a mixture of coccoid and bacillary forms. Only after several subcultures on cystine-blood-agar medium did the organism grow on serum-glucose-cystine agar. The organism was found to be nonmotile.

Using both the original beaver and muskrat material and cystine-blood-agar subcultures, the various usual media were tried for growth. No growth was obtained in beef-infusion broth, nutrient broth, litmus milk, Kleigler's iron agar, gelatin, Loeffler's medium, or chocolate tellurite. Slight acidity was noted in dextrose, saccharose, and lactose serum media.

The organism grew readily in the yolk sac of the developing chick embryo on direct isolation from beaver or guinea pig material. The maximum growth in the yolk sac was obtained after six days of incubation at 37° C., although the organism could be observed in the smears of the yolk sac as early as 48 hr. after inoculation. In that respect the developing chick embryo presents an obvious advantage over guinea pig for the initial isolation of the organism, providing, of course, that the starting material is free of contaminants.

Serological Characteristics of the Organism

Sera from several guinea pigs (total 11) inoculated with the original beaver material and succumbing to the disease on the seventh day or later were tested for the presence of agglutinins for *P. tularensis*. One of these guinea pigs did not develop any obvious signs of illness until the 20th day when it was exsanguinated. *P. tularensis* was isolated from all these animals. Serological results, however, in all cases, were negative.

It is also of interest to mention that the antigens prepared from the first cystine-blood-agar subcultures in all instances failed to react with our standard *P. tularensis* antiserum. However, after third subculture on the above medium the organism was readily agglutinable by the standard serum.

Rabbits immunized with formalin-killed antigens prepared from later subcultures of both the beaver and muskrat strains developed agglutinins to approximately the same titer for standard *P. tularensis*, beaver, and muskrat strains. Cross agglutination and absorption tests failed to distinguish between the three strains.

Discussion

The present limited survey provides positive evidence of tularemia infection among the beaver and muskrat population of Ontario. The infection, geographically speaking, is apparently widespread, since the organism was isolated from the animals obtained from three widely separate areas (Patricia, Long Point, and Gananoque districts). Although, to the authors' knowledge, the occurrence of tularemia in beavers and muskrats in Ontario is being reported for the first time, it is unlikely that spontaneous tularemia among these animals is a new phenomenon, since several similar earlier epizootics have been observed by trappers and fur traders. This view is further supported by the indirect evidence provided by Johns (8) who in 1933 reported three human cases of tularemia all associated with the handling of trapped muskrats.

The precise mode by which *P. tularensis* is maintained and disseminated under natural aquatic conditions so far remains unascertained. Occurrence of *P. tularensis* in natural waters (4, 5, 9, 10, 11, 12, 14, 15, 18) suggests the possibility of the infection being spread through the medium of water. Recent extensive work by the Rocky Mountain Laboratory workers (13) provides strong evidence that the water–mud mixture at the bottom of ponds and streams may act as a medium for the proliferation of the organism under natural conditions. Should further work support these findings the question of tularemia in semiaquatic animals will assume greater significance not only from the economic aspect but also from the public health standpoint. Human infections acquired through contact with contaminated water have been reported in the United States (6), and abroad (4, 9, 11, 14, 16, 19). Evidence of the danger to man of contracting the disease through handling infected carcasses is supported by the fact that there are over 100 human cases on record in North America where infection was traced to contact with infected beavers or muskrats (7, 13). The recent serological survey by Allin (2) demonstrates that it is not unusual to find agglutinins for *P. tularensis* in the blood of Indians in Northwestern Ontario, many of whom are trappers by trade.

Acknowledgment

The present investigation was made possible through the cooperation of various members of the Department of Lands and Forests of Ontario. The authors are greatly indebted to Miss V. M. Crossley, Central Laboratory,

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THE STUDY OF LIVER FUNCTION IN DOGS[1]

By Allan G. Gornall and Charles J. Bardawill[2]

Abstract

Three years experience with the study of liver function in dogs given carbon tetrachloride has led to the following conclusions. The most generally useful test of disturbed liver function has been a modified bromsulfalein excretion test in which a dose of 10 mgm. per kilogram is used and the blood sampled 15 min. later. The serum "alkaline" phosphatase level, in the absence of extrahepatic biliary obstruction, is a sensitive index of liver damage in dogs. The albumin/euglobulin ratio decreases during hepatotoxin administration and usually remains low for a time during hepatic regeneration. The degree of lowering gives a useful indication of the clinical condition of the animal and correlates best with the histological findings. Serum bilirubin was an insensitive indication of liver damage and urinary bilirubin and urobilinogen studies were of no value under our conditions. The plasma prothrombin time was increased only in the early acute phase of liver injury. Certain empirical tests for altered serum proteins, thymol turbidity, zinc sulphate, and 13½% sodium sulphite can be applied to dogs but are much less sensitive than they are in man. Amino acid tolerance tests gave little evidence of being useful and the intravenous galactose tolerance test was of no value in dogs.

The value of biochemical data and the relative merits of liver function tests in assessing the condition of a patient's liver have not been fully elucidated. One approach to the problem is to produce in a suitable experimental animal a known type of lesion and to observe the manner in which the different tests reflect the changes that occur. A number of workers (3, 24, 11, 31, 49, 50, 23) have employed this method with dogs.

We elected to use young, adult, mongrel dogs in order to be able to apply certain tests on a scale comparable with tests on humans and because the digestive faculties of this species appear to be omnivorous (37). These animals received diets of about 900 calories daily containing either a relatively low (8.5%) or moderately high (35%) proportion of protein on a dry weight basis. Most of the dogs chosen were females which made it possible, after the perineum had been split, to collect urine by catheterization. Liver injury was produced by the administration twice weekly through a stomach tube of 15 ml. of a 33% (v/v) solution of carbon tetrachloride in corn oil. It has been found (14, 29, 22) that one or two doses using this amount of the hepatotoxin (5 ml.) produces a cloudy swelling of the parenchymal cells with centrilobular necrosis. If the toxic agent is stopped after a few doses these pathological changes regress rapidly, but if it is continued there results a diffuse hepatic fibrosis which after several months may occasionally progress spontaneously to a fatal termination. Twenty-five dogs in all were followed during periods of carbon tetrachloride administration and recovery, some of them for nearly three years. Unless otherwise stated the tests were performed 48 hr.

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Contribution from the Department of Pathological Chemistry, University of Toronto, Toronto, Ont.

[2] *Medical Research Fellow, National Research Council of Canada.*

after the last dose during periods when the toxic agent was being given. The results of the different tests that were applied are summarized in the following section. Biopsies were obtained at various stages of the investigation and the animals which survived were sacrificed for autopsy. Particular emphasis is placed on certain differences in response that exist between the dog and the human species.

Functions Tested and Results Obtained

A. Secretory and Excretory Functions

1. Bilirubin

Total bilirubin has been measured in the serum by the method of Malloy and Evelyn (36) and in the urine by methylene blue titration (15).

Normal dog serum has practically no color; bilirubin seems to be excreted more efficiently in this species than in man (9), the serum concentration being less than 0.2 mgm. per 100 ml. In dogs receiving carbon tetrachloride the changes noted have been similar to those described by Hoffbauer (23). With damage sufficient to raise the bromsulfalein retention to 30% and higher the serum bilirubin increased usually to between 0.4 and 0.8 mgm. %. In two instances, after relatively few doses of hepatotoxin, the levels reached 2.3 and 3.7 mgm. % with bromsulfalein retentions of 54% and 80% respectively. Both of these dogs died shortly afterward, apparently from acute hepatic failure; the livers at autopsy showed diffuse yellow staining with widespread hemorrhagic areas. In acute liver injury the serum bilirubin may reach a level of 2.0 mgm. % or over with subsequent recovery but in most cases such values carry a poor prognosis.

The urine of dogs receiving carbon tetrachloride was found to contain bilirubin in moderate amounts (+ to ++) when the serum bilirubin was around 0.5 mgm. %. The renal threshold for this pigment is probably lower than in humans though the kidneys may not be normal during carbon tetrachloride administration.

2. Urobilinogen

Urine was tested for urobilinogen in most instances by the semiquantitative method of Watson *et al.* (52). Although a study of urobilinogen excretion is of considerable value in human patients with liver disease, it has been of little use in our experiments. There are several possible explanations for this difference.

Kelly *et al.* (26) emphasize the importance in Watson's method of adding the sodium acetate about 15 sec. after the Ehrlich's reagent, if color due to nonurobilinogen substances is to be minimized. In dog urine this interference may be very marked and both time and dilution factors must be rigidly controlled. Even so we have found that the longer quantitative method (44) sometimes showed only traces of urobilinogen in specimens that had given a strong positive test by the shorter procedure, a fact which may be due to the presence of dextrourobilinogen (42).

In the urine of normal dogs 'apparent' urobilinogen levels of 0–2 mgm. % have been noted. During carbon tetrachloride administration the quantities sometimes increased, but more often fell, frequently to zero. With no evidence of appreciable bilirubin retention this change has been attributed to a depression of bacterial activity in the intestine, thus interfering with the reduction of bilirubin to urobilinogen. This view receives support from the observation that the highest levels (around 6 mgm. %) occurred soon after withdrawal of the toxic agent. It is necessary to interpret urobilinogen studies with caution in patients receiving any medication which may affect the bacterial flora of the intestine.

3. Bromsulfalein Excretion

Macdonald (30) studied the rate at which bromsulfalein is removed from the blood in humans, and recommended a test dose of 5 mgm. per kilogram body weight. We have carried out a similar investigation in dogs and it is quite apparent that in this species the dye disappears more rapidly than it does from human blood. In Fig. 1 are shown the results obtained following the injection of 5 mgm. and 10 mgm. per kgm. doses in dogs. Curves *B* and *H* give a comparison of the two doses in the same animal with liver injury.

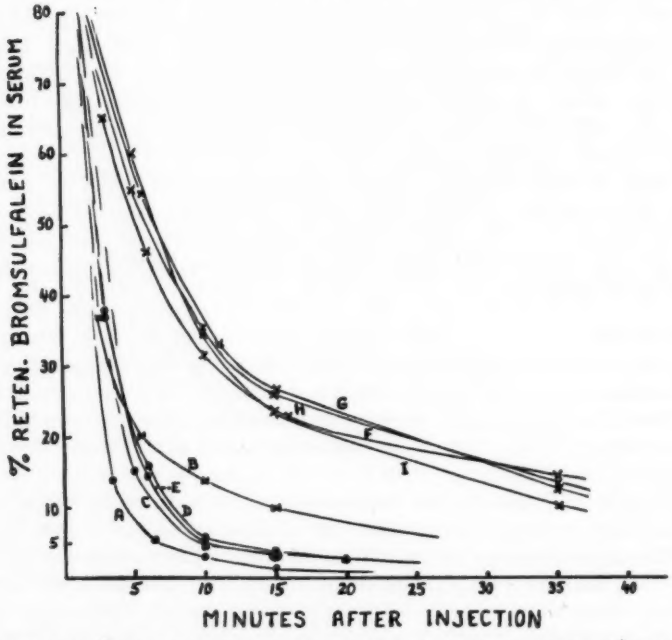

FIG. 1. Rate of disappearance of bromsulfalein from the serum following injection of a 5 mgm. per kgm. dose – Curve *A* (normal dog), Curve *B* (liver injury); and a 10 mgm. per kgm. dose – Curves *C*, *D*, and *E* (normals), Curves *F*, *G*, *H*, and *I* (liver injury).

Repeated tests on normal dogs revealed that 15 min. after a dose of 10 mgm. per kgm. the amount of bromsulfalein retained in the serum was less than 6%.

Procedure

Commercial bromsulfalein was diluted with 0.9% saline to a concentration of 1% (w/v). One milliliter of this solution was injected into the jugular vein for each kilogram body weight. (In an animal grossly over- or underweight an estimate of the "ideal" weight is preferred.) Blood was withdrawn from the opposite side 15 min. later.

Into each of two colorimeter cuvettes 0.5 ml. of serum was pipetted; to one was added one drop of 5% hydrochloric acid and to the other one drop of 10% sodium hydroxide. Each was then diluted with 9.5 ml. of 0.9% saline and mixed. The percentage transmission (or optical density) of the alkaline tube was read at 565 mμ in an Evelyn photoelectric colorimeter using the acid tube as the "blank".

A standard curve was prepared using 0.1 to 0.7 mgm. of bromsulfalein per 100 ml. of final alkaline solution. The optical density of these tubes represented 10 to 70% retention for either a 5 mgm. per kgm. dose, where a 1 to 10 dilution is used and 10 mgm. of dye per 100 ml. of serum is taken as 100% retention, or a 10 mgm. per kgm. dose, with a 1 to 20 dilution and complete retention assumed to be 20 mgm. %.

Mills and Dragstedt (38) and Brauer *et al.* (5) have studied the mechanism of bromsulfalein excretion using dogs, and other investigators (11, 31, 49, 50, 23) have indicated the usefulness in this species of the bromsulfalein excretion test. With the modification employed in our experiments it has been a sensitive index of liver damage. Following a single dose of carbon tetrachloride, the response to the test shows the percentage retention increasing to a maximum in about 24-48 hr. with a return to normal or near normal values in one to two weeks. During periods when toxin was being administered at intervals of three or four days, the tests have therefore been made 48 hr. after the last dose. Under these circumstances, although one cannot be certain, it seems reasonable to assume that the results obtained represent a rough mean of the prevailing disturbance. The effect of carbon tetrachloride on hepatic function measured in this way is illustrated in the paper which follows.

In Table I are listed representative data from our studies comparing the bromsulfalein excretion test, the serum alkaline phosphatase level and the serum protein fractions. The results are those obtained before, in the middle, at the end, and two to three months after different series of carbon tetrachloride administration. The diet and the number of doses in each series are indicated. Where there was some variation in the results an average of a number (indicated in parentheses, Column 6) of points is recorded. It can be noted from this table that the bromsulfalein retention reflects, probably with fair accuracy, the disturbance of liver function that one might expect an hepatotoxin to produce.

TABLE I

Dog	CCl_4 series	Total doses in series	Dietary protein	Time in relation to period on CCl_4	Retention bromsulfalein, %	Serum alkaline phosphatase, units	Albumin / Globulin	Albumin / Euglobulin	Damage assessed histologically
1	1st	25	High	Before	3	6	$\left(\frac{3.1}{3.0}\right)$	—	
				Middle	19 (4)	12	$\frac{3.5}{2.0}$	—	
				End	26 (2)	9	$\frac{3.6}{2.2}$	—	
				3 Months after	5	4	$\frac{3.7}{2.6}$	—	±
	2nd	9	High	Before	4	3	$\frac{3.3}{1.9}$	—	
				End	24	14	$\frac{3.1}{2.8}$	—	++
2	1st	53	High	Before	9	5	$\left(\frac{3.9}{2.4}\right)$	—	
				Middle	39	11	$\frac{3.6}{3.0}$	—	++
				End	40	20	$\frac{4.0}{2.1}$	—	+++
				1 Month after	15	9	$\frac{3.9}{3.7}$	—	
	2nd	26	High	End	14	10	$\frac{3.9}{2.3}$	—	++
				3½ Months after	11	3	$\frac{4.4}{2.3}$	—	
	3rd	16	Low	Before	4	3	—	$\frac{3.2}{1.1}$	
				Middle	13	4	—	$\frac{3.0}{1.3}$	
				End	37	22	—	$\frac{3.0}{1.5}$	
				3 Months after	10	5	—	$\frac{2.9}{2.1}$	±
	4th	16	High	Before	11	3	—	—	
				Middle	21	17	—	$\frac{3.2}{1.8}$	
				End	30	23	—	$\frac{2.7}{1.7}$	
				2 Months after	5	4	—	$\frac{3.1}{1.5}$	
5	1st	22	High	Before	5	5	$\left(\frac{2.7}{2.9}\right)$	—	
				Middle	24 (4)	20 (4)	$\frac{3.3}{3.0}$	—	
				End	31	25	$\frac{3.2}{3.2}$	—	
				3 Months after	6	4	$\frac{4.1}{2.2}$	—	
	2nd	9	High	Before	3	4	$\frac{3.8}{1.9}$	—	
				End	50	28	$\frac{4.2}{2.0}$	—	± (10 days after)
	3rd	26	High	End	19	16	$\frac{3.9}{2.2}$	—	±
				3½ Months after	8	3	$\frac{3.9}{2.3}$	—	

TABLE I—*Continued*

Dog	CCl_4 series	Total doses in series	Dietary protein	Time in relation to period on CCl_4	Retention bromsulfalein, %	Serum alkaline phosphatase units	Albumin / Globulin	Albumin / Euglobulin	Damage assessed histologically
	4th	16	Low	Before	5	—	—	$\frac{3.2}{1.0}$	
				Middle	50	37	—	$\frac{2.9}{1.3}$	
				End	41	25	—	$\frac{2.9}{1.5}$	
				2 Months after	8	16	—	$\frac{2.6}{1.6}$	0
10	1st	46	High	Before	5	3	$\left(\frac{3.0}{3.3}\right)$	—	
				Middle	33 (4)	10 (4)	$\frac{2.8}{2.8}$	—	++
				End	38 (2)	16 (2)	$\frac{3.1}{2.8}$	—	+++
				1 Month after	—	—	$\frac{3.0}{3.3}$	—	
	2nd	26	High	End	13	7	$\frac{3.0}{3.2}$	—	++
				3½ Months after	7	3	$\frac{3.6}{2.4}$	—	
	3rd	16	Low	Before	10	—	—	$\frac{2.8}{1.4}$	
				Middle	37	12	—	$\frac{2.3}{2.2}$	
				End	22	10	—	$\frac{2.1}{2.4}$	
				3 Months after	10	8	—	$\frac{2.7}{2.2}$	+
	4th	16	High	Before	7.5	3	—	—	
				Middle	29	9	—	$\frac{3.0}{2.2}$	
				End	24	8	—	$\frac{2.5}{1.9}$	
				2 Months after	18	3	—	$\frac{2.6}{1.5}$	
11	1st	50	Low	Before	4	2	$\frac{3.9}{2.1}$	—	
				Middle	38 (3)	43 (2)	$\frac{4.0}{2.3}$	—	+++
				End	43	83	$\frac{3.2}{2.7}$	—	
				3 Months after	7	3	$\frac{3.1}{2.6}$	—	
	2nd	16	High	Before	6	—	—	$\frac{3.2}{1.1}$	
				Middle	59 (2)	65	—	$\frac{3.0}{1.3}$	
				End	62	72	—	$\frac{2.8}{1.6}$	+
				3 Months after	8	5	—	$\frac{2.7}{1.5}$	
	3rd	16	Low	Middle	25 (2)	16	—	$\frac{2.2}{1.7}$	
				End	50	46	—	$\frac{2.2}{1.9}$	
				2 Months after	11	15	—	$\frac{2.4}{2.1}$	

TABLE I—*Concluded*

Dog	CCl_4 series	Total doses in series	Dietary protein	Time in relation to period on CCl_4	Retention bromsulfalein, %	Serum alkaline phosphatase, units	Albumin / Globulin	Albumin / Euglobulin	Damage assessed histologically
12	1st	50	Low	Before	4	2	4.0 / 2.8	—	
				Middle	27 (2)	28	3.8 / 3.0	—	
				End	46	22	2.8 / 4.0	—	+++
				3 Months after	12.5	13	2.5 / 4.6	—	
	2nd	16	High	Before	12	—	—	3.2 / 1.0	
				Middle	48 (2)	30	—	3.1 / 1.9	
				End	45	33	—	2.7 / 2.5	
				3 Months after	15	12	—	2.6 / 1.8	++
	3rd	14	Low	Middle	48 (2)	28	—	2.0 / 1.8	
				End	49	37	—	1.8 / 2.8	
				2 Months after	54	49	—	1.7 / 2.9	+++
15	1st	50	Low	Before	3	3	4.3 / 2.1	—	
				Middle	14 (3)	16	4.2 / 2.0	—	
				End	35	26	4.2 / 1.8	—	+
				3 Months after	6	3	3.4 / 2.5	—	
	2nd	16	High	Before	6	—	—	3.7 / 1.0	
				Middle	19 (2)	12	—	3.7 / 1.0	
				End	45	21	—	3.6 / 1.3	
				3 Months after	7	3	—	3.2 / 1.2	±
	3rd	16	Low	Middle	11 (2)	10	—	2.5 / 2.0	
				End	10	14	—	2.6 / 1.9	
				2 Months after	9	4	—	2.9 / 2.0	
24	1st	29	Low	Before	4	—	—	3.2 / 1.0	
				Middle	49 (2)	7	—	3.1 / 1.3	
				End	60	11	—	2.9 / 1.0	
				2½ Months after	11	4	—	—	
	2nd	16	High	Before	11	4	—	—	
				Middle	33 (3)	6	—	3.1 / 1.2	
				End	37	18	—	2.7 / 1.3	
				2 Months after	27	4	—	2.8 / 2.1	

4. Serum Alkaline Phosphatase

The method of Shinowara *et al.* (46) has been employed, phosphate being measured by Gomori's (16) procedure.

In humans it has been noted that marked increases in serum "alkaline" phosphatase usually arise from an obstructive lesion of the bile capillaries or ducts, smaller elevations being seen at times in hepatocellular disease. Tanturi *et al.* (50) have shown that very high phosphatase values occur in dogs with biliary obstruction, but Freeman *et al.* (13) and others (11, 49, 10) have noted that in this species high levels are also found with and constitute a useful test for parenchymal cell damage.

"Alkaline" phosphatase levels on normal dog sera fell in the same range (2-8 units) as humans, which confirms the findings of others. Dogs given two doses of carbon tetrachloride showed increases of two–seven times their normal values, the amount usually returning to within normal limits in one–two weeks. When the toxin was continued, eight out of 13 animals had levels of 25-85 units within a month, the others exhibited smaller increases or a slower rise. Changes in the serum phosphatase are recorded in Table I and are illustrated in the paper which follows.

In Fig. 2 the results of the bromsulfalein excretion test are plotted against serum phosphatase values. It can be seen that there is a rough correlation.

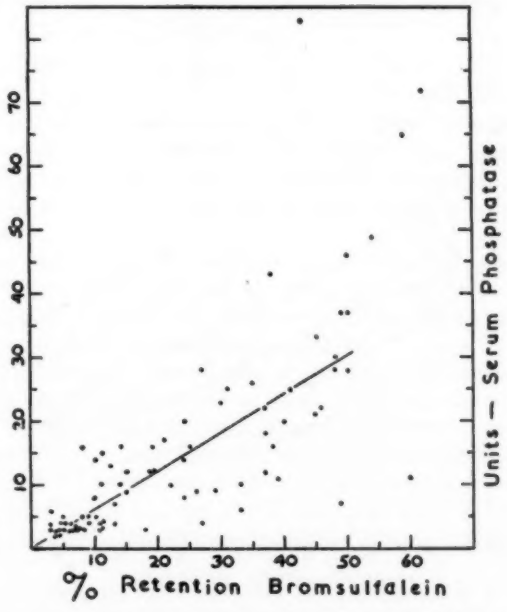

FIG. 2. Percentage retention of bromsulfalein plotted against units of serum alkaline phosphatase, showing the degree of correlation between these two tests.

As retention figures increase up to 50%, phosphatase values tend to rise to about 30 units. When bromsulfalein retention is very marked, however, there may be a wide spread in phosphatase levels from 7-83 units.

B. Metabolic Functions

1. Carbohydrate Metabolism

(a) Galactose Tolerance

In its intravenous modifications the galactose tolerance test has been considered moderately sensitive and reliable in patients with liver disease. Drill and Ivy (11) were unsuccessful, however, in applying it to dogs. Colcher *et al.* (7) have described a modification of the test for humans in which a "galactose removal constant" expresses the rate of disappearance of the sugar. This variation they regard as equal to the bromsulfalein test in sensitivity. Their procedure has been applied to dogs in the following way.

For each kilogram body weight of the animal 1 ml. of 50% (w/v) galactose solution, at 37° C., was injected into the jugular vein. At 15 and again 45 min. following the injection, blood samples were obtained. Galactose in the serum was determined, after removal of fermentable sugars by yeast, by the reduction method of Schales and Schales (43).

Typical results are illustrated in Fig. 3 for normal dogs, and dogs with moderate, diffuse parenchymal cell damage. There was no significant

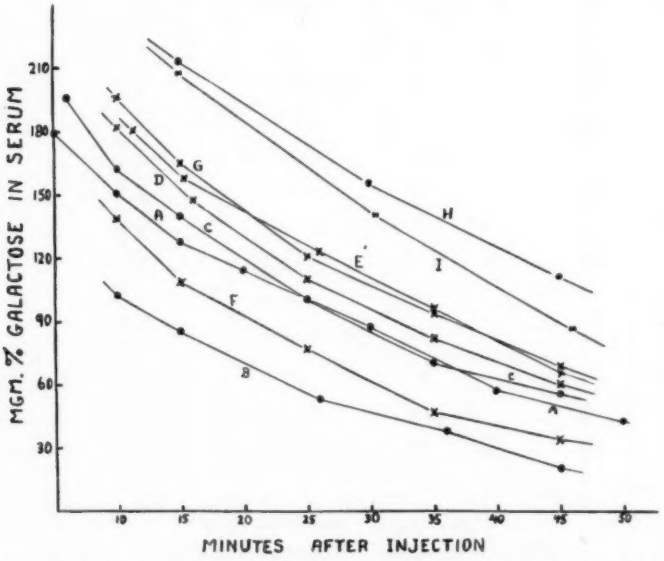

FIG. 3. Rate of disappearance of galactose from the serum following injection of 0.5 gm. per kgm. – Curves *A*, *B*, *C* (normal dogs), Curves *D*, *E*, *F*, *G* (liver injury); and of a 0.75 gm. per kgm. dose – Curve *H* (normal) and Curve *I* (liver injury).

difference between the galactose removal constants obtained for normal and abnormal dogs. Increasing the quantity of galactose injected failed to give better separation of the two groups. One must conclude that galactose utilization is more readily effected by or less dependent upon (4) the liver in dogs than in man.

2. Protein Metabolism

(a) Albumin and Globulins in Serum

In our earlier studies the proteins of serum were fractionated into albumin and total globulins by the method of Howe (25). Later we followed the procedure of Majoor (35) separating at 1 in 20 dilution albumin, pseudoglobulin, and euglobulin. Total globulins were precipitated with sodium sulphate at a final concentration of 26% but, from fractionations similar to Majoor's, we preferred a final concentration of 18% in separating euglobulins. Protein estimations were according to Gornall *et al.* (17).

Tumen and Bockus (51) and Post and Patek (39) found that hypoalbuminemia is the most consistent alteration of the serum proteins in patients with chronic advanced liver disease. Increases in the globulins, though present, were less constant. The protein pattern in dogs is quite similar to that in humans (53) and hepatectomy causes a decrease mainly in albumin, euglobulin, and fibrinogen (2). Kerr *et al.* (27) observed low albumin values in dogs with phosphorus poisoning and Elman and Heifetz (12) made similar observations on dogs fed low protein diets.

In our initial studies it was found that the infection and inflammation following operation, particularly the splitting of the perineum, produced a serious disturbance of the serum protein pattern. Those ratios in parentheses in Column 7, Table I, are attributed to this effect. Dogs that are well fed and apparently normal have a total serum protein concentration averaging slightly less than humans, with an albumin/globulin ratio of about 2.0 by Howe's method.

With the fractionation procedure used later, the serum proteins were made up of about 55% albumin (3.5 gm. %), 30% pseudoglobulin (2.0 gm. %), and 15% euglobulin (1.0 gm. %). This result differs from that for humans mainly in showing a lower proportion of euglobulin.

The results in Column 9, Table I, and in the paper which follows, show the effect of carbon tetrachloride administration. The first change was usually a rise in euglobulins with a less rapid decrease in albumin; the two curves cross in some instances. Pseudoglobulin showed a rather indefinite tendency to decrease slightly. The albumin/euglobulin ratio usually decreased sharply during the period of injury and sometimes continued to do so for a time after the toxin was stopped. Its return to normal values probably indicates complete recovery.

The albumin/euglobulin ratio does not correlate directly with either the bromsulfalein retention or phosphatase levels except that it is invariably low when these values are very high. It has been our experience that the animals

with lowest albumin/euglobulin ratios were those that appeared to be in the poorest state clinically and showed evidence of more severe liver derangement histologically.

(*b*) *Empirical Tests for Altered Serum Proteins*

The observation of Hanger (20) and others that cephalin cholesterol emulsions are flocculated by normal dog serum has been confirmed. Limited success has been attained, however, in applying the thymol turbidity test (32), zinc sulphate test (28), and 13½% sodium sulphite test (8). The turbidity which develops with each of these tests in the presence of altered serum proteins has been measured in terms of the barium sulphate standard curve described by Shank and Hoagland (45), readings being made with the Evelyn colorimeter using a 660 mμ filter. The results are expressed as "units" of turbidity.

(*i*) *The Thymol Turbidity Test*

The buffered thymol reagent prepared according to Maclagan had a pH of 7.6 $\pm$ 0.05 and owing to changes which affected its sensitivity it was prepared fresh every two weeks. One-tenth milliliter of serum was mixed with 6 ml. of reagent. Turbidity was read after 30 min. and the tubes were examined for flocculation after 18 hr. Recant *et al.* (41) state that this test is negative in normal animals and Brieger and Friedman (6) report changes in rabbits given carbon tetrachloride.

In normal dogs the turbidity readings varied from 0.2 to 1.2 and averaged 0.5 units at 30 min.; in no case was flocculation observed after 18 hr. When liver injury was produced with carbon tetrachloride there was a tendency for the turbidity to become greater, averaging 2.0 units, but the highest value obtained was only 4 units in a dog with marked chronic liver injury and a high level of euglobulins. In the early, acute phase there were occasional higher values associated with lipemic sera. This test can be used in dogs but is much less sensitive than it is in humans.

(*ii*) *The Zinc Sulphate Test*

Kunkel's method was followed, 0.1 ml. of serum being treated with 6.0 ml. of the reagent and the results read after 30 min.

Normal values in these dogs never exceeded 0.4 units, but again the test was much less sensitive than in humans, showing values averaging 1.3 units in dogs with liver injury and a maximum rise to only 2.5 units in a dog with marked hepatic damage and increased euglobulins.

(*iii*) *The 13½% Sodium Sulphite Test*

Dauphinee and Campbell have noted that increased serum globulins in patients with liver disease occur mainly in the euglobulin fraction and can in large part be accounted for by the portion that precipitates with 13½% sodium sulphite. For our studies 6 ml. of this salt solution were added to 0.2 ml. of serum and the turbidity was read 30 min. later.

In normal dogs turbidity readings were less than 2.0 units. The average result in liver damage was 2.7 units and the highest result obtained was 6.0 units, again in an animal with a marked increase in serum euglobulins. This test also is less useful in dogs and would account for only a small portion of the increased euglobulins.

(*c*) *Amino Acid Tolerance Tests*

(*i*) *Tyrosine Tolerance*

Bernhart and Schneider (1) have compared the efficiency with which tyrosine is handled by normal humans and patients with liver disease, claiming that it is more sensitive than the bromsulfalein test. Hier (21) gave oral tyrosine to normal dogs and the blood level increased from 1.65 to 9.75 mgm. % in three hours.

We have given fasting dogs a mixture of 6 gm. of tyrosine suspended in 180 ml. of milk, which they drank readily. At intervals thereafter blood was sampled for tyrosine estimations. The method of Bernhart and Schneider has been adapted to the Evelyn colorimeter with the following modifications: (*a*) Color density was measured with a 490 mμ filter; (*b*) 1.0 ml. of 7 *N* sulphuric acid was found to give clearer solutions than 0.5 ml.; and (*c*) eight minutes were required for final color development.

Fasting "tyrosyl" values in six normal dogs and three humans ranged from 1.1 to 1.9 mgm. %. Fasting levels in three patients with liver disease were 2.1, 3.1, and 3.2 mgm. %. In three dogs given carbon tetrachloride the fasting value for two 48 hr. later was above 2 mgm. %, the third was normal six days after the toxin. Fig. 4 illustrates the change in blood "tyrosyl"

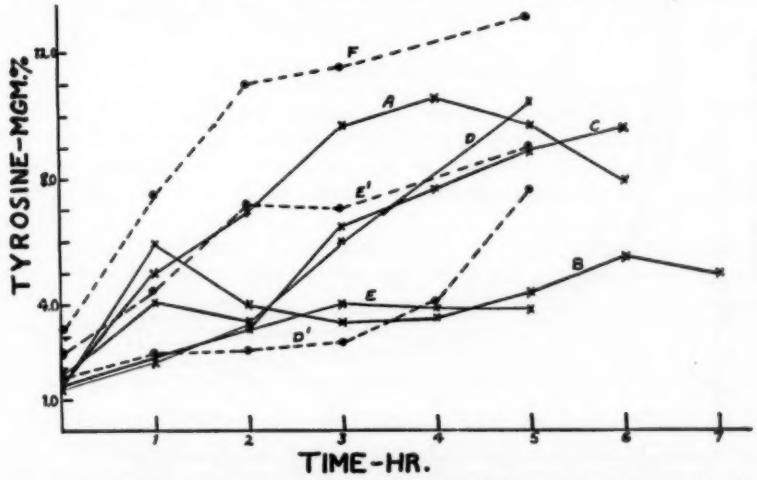

FIG. 4. Changes in blood tyrosine levels following ingestion of 6 gm. of tyrosine. Curves *A*, *B*, *C*, *D*, *E* are from normal dogs, Curves E^1 and *F* were obtained 48 hr. after a single dose of carbon tetrachloride, Curve D^1 was obtained six days after the toxin. ——— Normal dogs. — — — — Liver injury.

levels during five to seven hours following ingestion of tyrosine by normal dogs and dogs given carbon tetrachloride. Curves *E* and *E'* show results on the same dog before and 48 hr. after the toxin. This test appears to show some changes in dogs, but our data do not permit a satisfactory estimate of its reliability.

(ii) Arginine Tolerance Test

Shvarts and Krynskii (47) observed diminished excretion of urea following injection of arginine in patients with liver disease.

We have investigated the rate of removal of arginine in dogs. A solution of 1.5 gm. of L-arginine hydrochloride in 10 ml. of water was neutralized and injected intravenously and the blood levels followed over a four-hour period. Arginine was determined by Macpherson's (34) modification of the Sakaguchi reaction adapted to a tungstic acid filtrate and measured in the Evelyn colorimeter using the 490 mμ filter. Recovery experiments were accurate to within $\pm$ 6%.

The results are illustrated in Fig. 5 where the arginine levels in two normal dogs are compared with those in three dogs with moderately severe liver damage. The data indicate no significant difference in the rate of removal

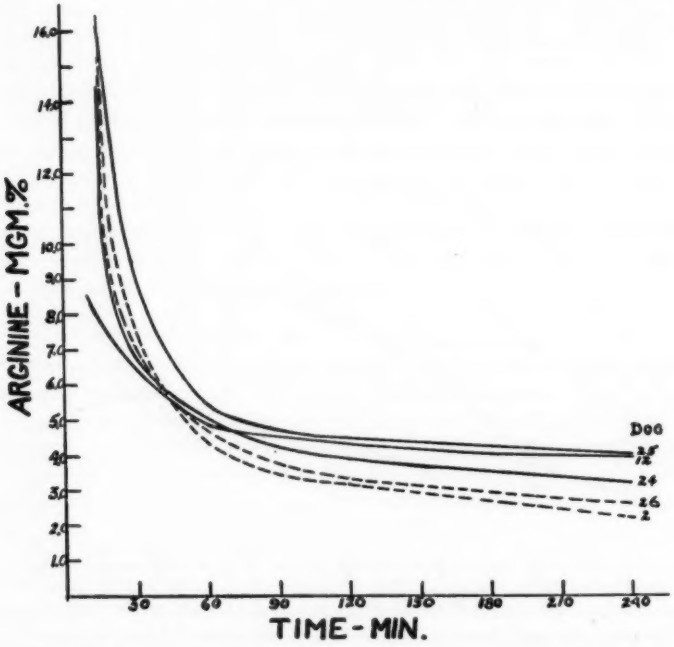

FIG. 5. Arginine levels in the blood following intravenous injection of 1.5 gm. of arginine. — — — — Normal (dogs 2, 26). ——— Liver injury (dogs 12, 24, 25).

of this amino acid in the two groups. Normal fasting levels varied from 1.6 – 2.2 mgm. per 100 ml. and the dogs with damaged livers fell within this range except for one value of 3.0 mgm. %. It can be noted however that four hours after the injection all animals with impaired liver function had slightly higher arginine levels than the normals.

(*d*) *Plasma Prothrombin*

The blood of dogs receiving carbon tetrachloride frequently showed delayed clotting and poor clot retraction. Prothrombin estimations were carried out on undiluted, oxalated plasma by a modification of Quick's procedure (40). To 0.1 ml. of plasma was added 0.1 ml. of thromboplastin (Difco) extract and after two minutes at 37° C. the mixture was treated with 0.1 ml. *M*/40 calcium chloride. Clot formation was detected by sweeping the mixture constantly with a wire loop, the first appearance of fibrin threads being taken as the end point.

Normal dog plasma showed prothrombin clotting times ranging from 7.4 – 8.6 sec. Five dogs showed increased prothrombin times ranging from 11 – 24 sec. following the administration of two doses of carbon tetrachloride. These levels returned to normal within 10 days and a second administration of two doses showed a less marked increase. Six dogs that had received the toxin for several months showed normal prothrombin levels even though the blood of one of these animals had a definitely prolonged, spontaneous clotting time. With the method we have used prothrombin estimations may, as has been suggested (48, 50) be a sensitive index of liver injury in dogs during the early acute phase, but within a short time the levels return to normal despite continued damage and the test is no longer of value.

C. Miscellaneous Observations

(*a*) Gornall and Hunter (19) found evidence with rat liver slices that the conversion of citrulline to arginine is the limiting step in the ornithine cycle of urea synthesis. Citrulline estimations (18) on the blood of dogs with hepatic damage showed no detectable increase following the injection of arginine.

(*b*) It was noted that during periods of toxin administration the female dogs, when allowed together in the exercising pen, commonly showed signs of being in heat, particularly by masculine mounting behavior. Facilities for steroid hormone studies were not available.

Pathological Features of the Liver Damage Produced

Attempts to obtain satisfactory biopsies with the needle punch technic were unsuccessful. It was necessary to restrict this aspect of the work to occasional wedge samples taken at laparotomy from the edge of the main liver lobe. With advanced changes in liver structure it is not certain that these samples would be representative, but combined with the gross appearance of the organ they gave useful information.

The pathological changes observed were similar to those reported by Gardner *et al.* (14). The earliest changes (after two doses) showed a marked cloudy swelling with varying degrees of congestion, fatty degeneration, and necrosis, the last being most prominent in the region of the central veins. After 30-50 doses of carbon tetrachloride the biopsies disclosed a progressive disorganization of the normal liver architecture with an increase in fibrous tissue and new lobule formation. Degenerative changes and necrosis were less in evidence while reorganization of liver structure probably indicated an improvement in circulation with a return of patent sinusoids and bile canaliculi.

Six to eight weeks following withdrawal of the toxic agent after about 50 doses, when hepatic function appeared normal in most of the animals, necrosis was absent, inflammatory reaction minimal, and a variable degree of fibrosis was present. Parenchymal cells in the liver of those dogs with appreciable fibrosis appeared in some cases to be mostly of the normal polyhedral type, but in others we have noted, as MacNider (33) described, that moderate to large proportions of the parenchyma were composed of a flattened, syncytial type of epithelial structure with imperfect cell differentiation and variations in size and staining properties of the nuclei.

The last column in Table I gives an estimate of the state of the liver based on the gross appearance and an examination of the biopsy specimens. The following system has been followed in grading the histological damage:

0 Liver parenchymal cells appear uniform in size and staining properties – areas of focal necrosis if rare are ignored.

± Slight variation in size of cells and nuclei but cytoplasm uniform and showing no degenerative changes.

\+ Moderate variation in size of cells and nuclei – cytoplasm uniform but may show fine granulation.

++ Moderate irregularity of liver cell cords with variations in size of cells and nuclei and variations in staining with either nuclear changes indicating degeneration *or* cytoplasm changes of moderate degree (more eosinophilic or basophilic than normal and with irregular staining).

+++ Marked variation in size and shape and staining of cells and nuclei with more marked nuclear degenerative changes (than ++ above) *as well as* widespread cytoplasmic changes consisting chiefly of eosinophilic clumping of cytoplasmic material and/or high degree of vacuolization in the cytoplasm.

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THE INFLUENCE OF DIETARY PROTEIN ON LIVER INJURY DUE TO CARBON TETRACHLORIDE IN DOGS[1]

By Charles J. Bardawill[2] and Allan G. Gornall

Abstract

Six adult dogs, previously exposed to carbon tetrachloride and allowed a recovery period of seven months, have been kept at different times on isocaloric low protein and moderately high protein diets. During each of these periods a series of 16 consecutive doses of carbon tetrachloride was administered. Liver function was tested fortnightly by means of the bromsulfalein excretion test, serum alkaline phosphatase, and serum protein fractionation. Biopsy specimens were also examined. There was a notable variation from one dog to the next and the only useful comparison was the response shown on the two different diets by each individual animal. Analysis of the data indicated that the acute phase of liver injury during carbon tetrachloride administration was accompanied by less derangement of function when the dogs were on a low protein diet. In the recovery phase the higher protein intake resulted in a somewhat more rapid restoration of function. It is suggested that some restriction of protein is probably desirable during the acute phase of any destructive liver lesion. Once recovery and regeneration set in generous amounts of protein in the diet will tend to hasten the repair process.

The influence of nutritional factors on the effect of hepatotoxins has been investigated extensively for many years; the subject has been reviewed recently for the halogenated hydrocarbons by Miller (22). The protective value of a generous caloric intake seems clearly established, as is the harmful effect of a diet high in fat when the protein level is low or lipotropic factors are deficient. The question of the optimum intake of protein in liver disease is not yet settled. On the one hand there is ample evidence that animals which have been starved (7) or depleted of protein, by diet, bleeding, or plasmapheresis (25), are much more susceptible to toxic agents. On the other hand one must note the striking deterioration produced by feeding a lean meat diet to Eck-fistula (12) or bile-duct ligated dogs, or to dogs with advanced experimental cirrhosis (4). While a level of dietary protein either below maintenance requirements, or excessively high, may under different conditions and for different reasons jeopardize the welfare of the animal, a more practical question is the relative value of a low and high protein intake within more tolerable limits.

The present study grew out of an attempt to evaluate biochemical data and tests of liver function in dogs exposed to carbon tetrachloride (see preceding paper). During these studies the first group of four animals was kept on the usual kennel diet (Dog Chum) consisting largely of meat. Because the degree of liver damage after 25–50 doses of hepatotoxin was not very great, a second

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[2] *Medical Research Fellow, National Research Council of Canada.*

group of four animals was placed on a low protein intake and given 50 consecutive doses of carbon tetrachloride. Comparing the results of the biochemical analyses, liver function tests, and biopsy sections of the two groups it was difficult to distinguish between them. Because of the importance attached by some (28, 10, 1, 27, 31, 32) to a high protein diet as a protective and therapeutic measure in liver disease we were stimulated to investigate more carefully the influence of dietary protein on experimental liver injury.

Our experimental animals were relatively young, adult dogs all of whom had recovered more or less completely from an early to moderate, diffuse, hepatic fibrosis produced over a period of 7–12 months by 50–79 doses of carbon tetrachloride. The recovery period was just over seven months. These animals were kept in individual cages and fed once a day at noon; twice a week they were allowed several hours in an outdoor exercising pen. All were active and appeared to be in good health.

For our *low protein* diet we made use of a commercial preparation designed for the feeding of weanling pigs.* It contained protein derived largely from vegetable sources (only 5% of the mixture being of animal origin) and was fortified with minerals and vitamins. To 2750 gm. of this "pig starter" we added a melt of 1 lb. (450 gm.)of lard plus 50 ml. of corn oil and mixed them thoroughly. Approximately 100 gm. portions of this mixture and of sucrose were stirred to a mush with water and fed to each dog daily. This amount was estimated to provide about 900 calories made up of 17 gm. of protein,** 20 gm. of fat, and 160 gm. of carbohydrate. The choline content was only moderate (130 mgm.)*** and it is probable that the methionine content was somewhat low.

The *high protein* diet consisted of a commercial dog food made largely from meat by-products but containing some mixed grains and added minerals and vitamins.† Fed in approximately 400 gm. (wet weight) quantities it was estimated to provide about 900 calories made up of 70 gm. of protein,* 20 gm. of fat, and 105 gm. of carbohydrate. This diet supplied 400 mgm. of choline daily.**

The dogs normally were kept on the high protein, kennel diet, but were placed on the low protein diet for at least three weeks before a study of the

* *Master Pig Starter—Toronto Elevators Ltd., Toronto, Canada.*

Ingredients (courtesy Mr. H. R. Cook):—Feeding tankage and fish meal (5%), soybean oil meal and/or linseed oilcake meal (7%), wheat shorts, wheat bran, alfalfa meal, wheat middlings (52%), ground barley, oats and wheat (33%), 1/2 of 1% iodized salt, manganese sulphate, feeding bone meal, fortified vitamin A and D fish oil.

Analysis:—crude protein (min.) 18%,
crude fat (min.) 3.5%,
crude fiber (max.) 8%.

** *Protein calculated from nitrogen analyses after exhaustive extraction with hot 70% alcohol, using the factor 6.25.*

*** *For this assay we are indebted to Miss Jessie Lang and Dr. C. C. Lucas of the Banting and Best Department of Medical Research.*

† *Frozen Dog Chum, Canada Packers Limited. Ingredients (courtesy Mr. J. A. Jack):—Beef lungs, tripe, brain, lips, hog stomach, and liver (66%). Wheat, barley, soyabean, beet pulp, carrots (30%). Edible tallow, sugar, salts, yeast, fish oil concentrates, bone meal (4%). Water added.*

effects of toxin was begun. All but one of the dogs included in this report ate either of the diets equally well and usually completely. Dog 15 was a smaller dog with a small appetite and ate about half portions of either diet. Carbon tetrachloride was administered as a 33% (v/v) solution in corn oil, 15 ml. of the mixture being given through a stomach tube twice weekly in the morning.

The functional state of the liver of each dog was estimated at fortnightly intervals by means of the bromsulfalein excretion test, the serum alkaline phosphatase, and changes in the serum protein fractions, albumin, pseudoglobulin, and euglobulin (for methods see preceding paper). During periods of toxin administration the tests were made always 48 hr. after the last dose was given.

The main plan of the experiment was as follows: Six dogs were divided as evenly as possible into two groups, making use of the fact that we had some previous knowledge of the eating habits of these dogs and their response to carbon tetrachloride. The first group (Dogs 2, 5, and 10) was placed on the low protein, and the second group (Dogs 11, 12, and 15) was kept on the high protein diet. After three weeks on these diets a course of 16 consecutive doses of the toxic agent was administered to each dog over an eight week period. Dog 5 developed anorexia on the low protein diet after three doses of carbon tetrachloride and for three weeks would accept only the meat diet. Two months after the toxin was stopped, this animal died from renal failure owing to large phosphate calculi in both pelvices. Dog 24 was placed on the low protein diet at the beginning of March and after three weeks received the first of 29 consecutive doses of carbon tetrachloride.

At the end of this part of the investigation it could be noted that, irrespective of the diet, there was a distinct difference in the response of individual dogs to the same course of carbon tetrachloride administration, a fact which is as obvious in dogs that have never received toxin before as in the study shown here. Taking the results collectively there was no possibility of distinguishing clearly between the group on the *low* or *high* protein diet, either during the period of toxin administration or the succeeding recovery period of two and one-half months on the same diet.

Faced with the difficulty of assessing the significance of the results in a comparison between groups of small population, the best expedient seemed to be to reverse the two groups as to diet and repeat the experiment. Following an additional recovery period of three months on the usual kennel diet, Dogs 11, 12, and 15 were placed on the low protein and Dogs 2, 10, and 24 were kept on the high protein diet. After a three week period a course of 16 doses of carbon tetrachloride was administered as before. All went well except that after 13 doses Dog 12 developed anorexia on the low protein diet and began to deteriorate rapidly after the 14th dose of toxin. Three days on a nutritious liquid diet fed by stomach tube produced a marked clinical improvement and the animal then ate the low protein diet voluntarily. It can be noted however that the liver function tests on this dog did not improve and

after two weeks the high protein diet was substituted. Eight weeks later the dog again refused to eat and died in four days, apparently as a consequence of hepatic failure. The liver showed far advanced, nodular cirrhosis.

Results

After completion of the study outlined it was possible to compare not only the responses of the two groups on the different diets, but also of each individual dog on the low and high protein regimens. Our results are shown in Figs. 1, 2, and 3 which have the following features in common:

(*a*) The abscissae show the period of the experiment, from October 1947 to February 1949.

(*b*) Each dose of carbon tetrachloride is indicated by a short vertical line along the abscissa for the different animals.

(*c*) The number of doses of toxic agent previously administered is indicated in Fig. 1 by the number of the first dose given to each dog in this series.

(*d*) In Figs. 1 and 2 the periods when the animals were on low protein intake are indicated by *short dashes* in the graph lines; *solid lines* (or long dashes) mean that the animal was receiving the high protein diet. In Fig. 3 the barred strips above the graphs signify periods of low protein intake.

(*e*) The arrows indicate biopsies, fork-tailed arrows mean autopsies.

Fig. 1 shows the degree of bromsulfalein retention in the serum 15 min. after injection of the dye. Of the dogs that had received carbon tetrachloride

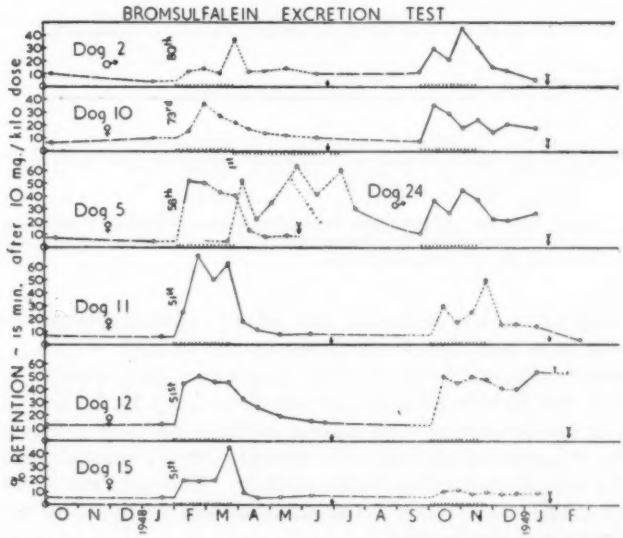

FIG. 1. Bromsulfalein retention during and following a series of doses (indicated by short vertical lines) of carbon tetrachloride. Periods on low protein intake, short dashes; on high protein diet, solid lines.

before, one in each group (10 and 12) still had a retention significantly above the normal range of 6% before the first series (January, 1948) of toxin administrations shown. Dog 24 was a new animal and received 29 doses of toxin in the first series; the probable result had only 16 doses been given is suggested by the dotted line. At the start of the second series Dogs 2 and 24 were still above normal and probably No. 12 also, but owing to an oversight this dog received carbon tetrachloride before the test was repeated following the recovery period.

Fig. 2 illustrates the results obtained using the estimation of serum alkaline phosphatase. All of the animals except one had returned to within the normal range of two–eight units several weeks before the experiment began.

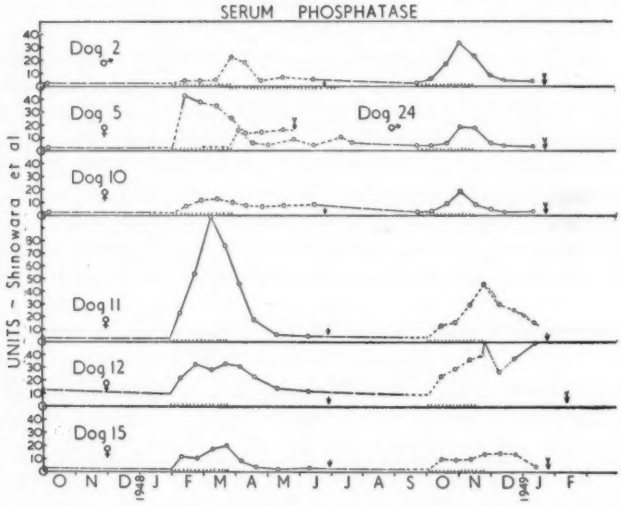

FIG. 2. Serum 'alkaline' phosphatase levels during and following a series of doses (indicated by short vertical lines) of carbon tetrachloride. Periods on low protein intake, short dashes; on high protein diet, solid lines.

Although Dog 12 was not rechecked just prior to readministering the toxin its level must have been close to the upper normal limit.

From the curves shown in Figs. 1 and 2 it is apparent that, taken collectively, Dogs 2, 10, 5, and 24 cannot be said to differ from Dogs 11, 12, and 15 under conditions of low protein or high protein intake. For each individual dog there seems, on casual inspection, to be in most cases a remarkable similarity between the results on the two different diets. The data can however be submitted to closer examination if one excludes Dogs 5 and 24, for which the conditions were not comparable.

For each of the other animals a horizontal base line was drawn at the best level of function attained in the recovery phases. Vertical lines were drawn

TABLE I

Dog	Test	Two month period	Area under curve		Difference		Ratio	CCl_4 area / Recovery area
			High protein	Low protein	On CCl_4	Recovery	High protein	Low protein
2	Bromsulfalein	On CCl_4	11.3	5.2	+ 6.1		2.76	0.90
		Recovery	4.1	5.8		−1.7		
	Phosphatase	On CCl_4	7.05	2.3	+ 4.75		2.82	0.56
		Recovery	2.5	4.1		−1.6		
10	Bromsulfalein	On CCl_4	8.0	8.2	− 0.2		1.36	2.10
		Recovery	5.9	3.9		+2.0		
	Phosphatase	On CCl_4	3.3	3.5	− 0.2		3.88	1.35
		Recovery	0.85	2.6		−1.75		
11	Bromsulfalein	On CCl_4	19.8	9.1	+10.7		3.39	1.32
		Recovery	5.85	6.9		−1.05		
	Phosphatase	On CCl_4	26.0	8.5	+17.5		2.45	0.77
		Recovery	10.6	11.1		−0.5		
12	Bromsulfalein	On CCl_4	14.6	14.0	+ 0.6		(3.01)	(1.87)
		Recovery	(4.85)*	(7.5)*		(−2.65)		
	Phosphatase	On CCl_4	9.2	9.3	− 0.1		(1.80)	(1.33)
		Recovery	(5.1)*	(7.0)*		(−1.9)		
15	Bromsulfalein	On CCl_4	7.8	2.35	+ 5.45		3.39	1.20
		Recovery	2.3	1.95		+0.35		
	Phosphatase	On CCl_4	5.2	3.1	+ 2.1		3.06	0.76
		Recovery	1.7	4.1		−2.4		
Averages					+ 4.66	−0.83 (−1.12)**	2.89 (2.79)**	1.12 (1.22)**

* *From one month period only.*
** *Including Dog 12.*

so as to mark off the two month period when the dogs received carbon tetrachloride and the succeeding two month recovery period. The area bounded by these lines and the curve above them was then measured with a compensating polar planimeter. The figures recorded in Columns 4 and 5 of Table I are in square centimeters and although of relative significance only represent a product of the severity and duration of deranged liver function. In Column 6 it will be seen that with minor exceptions the disturbance in function during carbon tetrachloride administration was greater on the high protein diet. The values in Column 7, however, though less definite, point to a somewhat better recovery on the high protein diet.

In order to relate the recovery phase to the severity of the acute phase the ratio of the areas in the two phases was calculated for the two different diets. If considerable derangement of function occurred, but recovery was rapid, a high ratio should result. If function was not so markedly impaired but recovery was slow, the ratio would be small. Column 8 shows that on the high protein diet the average ratio was 2.8 whereas, in Column 9, on the low protein diet the ratio averaged 1.2. Since this difference was as much the result of increased severity of damage on the high protein intake as of poor recovery on the low protein diet, one may conclude that a high intake of protein, at least in the form of meat, is undesirable during the acute phase of liver injury. In the recovery phase, when liver regeneration is occurring, a generous protein intake will tend to speed the return of normal function.

Fig. 3 illustrates the changes noted in the albumin, "pseudoglobulin" and "euglobulin" fractions of the serum proteins. The points shown represent the results as actually obtained; the lines are drawn in a few instances (e.g. Dog 12, Nov. 1948) through points calculated to correct for sudden changes in total protein which we attributed to shifts in water balance. During periods of carbon tetrachloride administration it is apparent that the euglobulin values tend to rise and the albumin levels to fall. Pseudoglobulins show less definite changes.

In *Fig. 4* the albumin/euglobulin ratios have been plotted for those dogs which could be compared on the two diets during and after carbon tetrachloride administration. The following comments can be made from the results shown.

In all the dogs there was a sharp downward trend in the A/E ratio during the acute phase of liver injury. For a varying period in the recovery phase the ratio may show no improvement and may fall still further. Sooner or later, if recovery occurs, the serum protein pattern begins to show a return to more normal proportions.

Although the fall in A/E ratio during hepatotoxin administration is about as severe on either diet there is a more definite tendency to hold this level during recovery and to return toward normal values on the high protein intake.

In June the animals appeared to be in such good health that the additional three months recovery period, during which they were all on the usual kennel diet, was assumed to be adequate to restore the protein pattern to A/E ratios

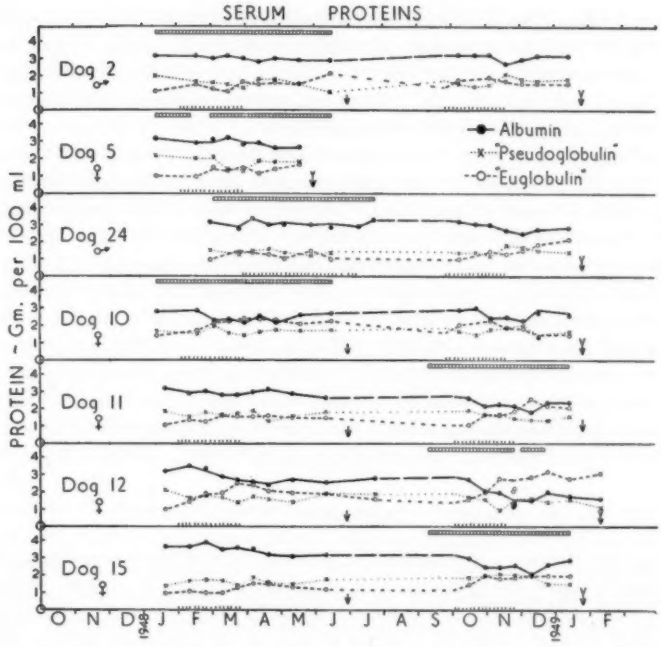

FIG. 3. Serum protein fractions during and following a series of doses (indicated by short vertical lines) of carbon tetrachloride. Periods on low protein intake indicated by barred strips.

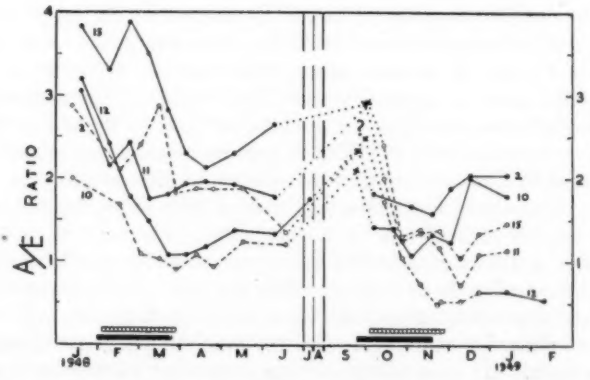

FIG. 4. Albumin/euglobulin ratios during and following eight week periods of carbon tetrachloride administration marked by a solid strip for the high protein and a barred strip for the low protein intake. Solid lines in the graphs indicate that the dogs were receiving the high protein diet, dashes indicate the low protein diet. The crosses represent an estimate of the ratio at the end of the first recovery period.

of 3 or over. Dog 12 was tested in July and showed improvement but a recheck of the levels in September was overlooked and the data suggest that a longer recovery period would have been better. If the ratios were subnormal at the start of the second series this may have had something to do with the poorer showing of the animals on low protein intake.

The A/E ratio reflects the derangement of hepatic function in the acute phase. It seems to demonstrate the increased need for protein during the recovery phase, possibly indicating the period when liver regeneration is proceeding most rapidly and the adequacy of nutrition at this time. The restoration of normal proportions of albumin and euglobulin can probably be regarded as evidence of complete recovery. With chronic liver damage the ratio tends to remain at subnormal values. In the first series shown in Fig. 4, Dogs 10 and 12 were most noticeably affected clinically and showed evidence of more marked liver damage at biopsy. In the second series Dog 12 went on to hepatic failure, whereas Dog 10 made a fairly good recovery. The A/E ratio was a somewhat more accurate index of the condition of these animals than either the bromsulfalein excretion or the serum phosphatase level.

Discussion

The data here presented are consistent with much that has been written about protein nutrition and the problems of liver injury and repair. Some confusion has arisen from attempts of others to apply results from one species to another, from growing to adult animals, or from one to another type of hepatic lesion. This discussion will be restricted mainly to work done on adult dogs exposed to carbon tetrachloride or chloroform.

Wang *et al.* (33) and Kade *et al.* (18) have provided evidence that a normal dog will maintain nitrogen balance on about one gram of protein per kilogram per day. The amount depends of course on the quality of the protein fed. Our animals weighed between 9 and 13 kgm. and the low protein diet supplied approximately 17 gm. of protein daily. This protein was not of highest quality, but the quantity seems to have made it adequate under normal conditions to maintain protein stores. There is evidence for this in the fact that the serum proteins did not fall until the toxic agent was administered, and the levels tended to improve during recovery periods on this diet. Lipotropic factors, minerals, and vitamins were undoubtedly higher in the meat diet but were apparently not deficient on the low protein regimen.

It is probable that during periods of carbon tetrachloride administration the low protein diet was barely or even less than adequate. On the average the dogs on this diet lost more weight than those on the high protein diet.

It has been known for many years (15, 20) that the halogenated hydrocarbons produce a marked loss of nitrogen in the urine when administered to dogs. Davis *et al.* (7, 6) found the loss greatest if the animal received no food after the toxin and showed that sugar alone greatly reduced the nitrogen wastage. Daft *et al.* (5) reported that dogs made anemic, but given iron, would regenerate hemoglobin very rapidly even when fasted, and excreted very little nitrogen.

Following liver injury by chloroform there was a marked loss of nitrogen but hemoglobin synthesis went on unimpaired. Miller *et al.* (25, 24, 26, 23) fed diets containing only 4–5 gm. of protein daily and found the liver injury from exposure to chloroform much greater as the fall in serum proteins reflected a depletion of protein stores. Loss of nitrogen following the toxin decreased as the protein stores diminished. They showed that a single protein feeding would protect depleted dogs from the anesthetic and among the amino acids methionine and cystine had an equal and specific effect. The action seemed to be due to a restoration of depleted sulphur; choline was not protective so the effect was probably not lipotropic. These authors found no alteration in liver function during protein depletion but such impairment has been reported (9), usually however under conditions which lead to the development of fatty livers (14, 21). Choline seems to have little effect on the fatty infiltration of the liver produced under suitable conditions by chloroform (9) or carbon tetrachloride (2) but acts by preventing fat deposition before and accelerating its removal after toxic liver injury. Kinsell *et al.* (19) have reported that choline has a marked anabolic, nitrogen-retaining effect in chronic, active liver disease.

The choline and methionine requirements of adult dogs are not known, and are undoubtedly interlinked. Our low protein diet provided daily about 130 mgm. of choline, the high protein diet around 400 mgm. Neither the methionine nor the betaine contents of the diets was determined, but the absence of any marked degree of fat vacuolation in paraffin sections of the biopsy specimens obtained during or after carbon tetrachloride administration would indicate that no serious lipotrope deficiency was present. It is possible that any increased requirement for these substances during periods of liver injury is offset to some extent by the presence of a relative excess of estrogenic hormone (11) or a depression of the bacterial flora in the intestine (16).

It has not been determined whether the dietary conditions which best protect an animal during periods of exposure to a toxic agent are the same as those which permit most rapid and effective repair of the liver injury. Bollman (3) reported less hepatic injury with a low protein, high carbohydrate diet when dogs were given carbon tetrachloride. Hoffbauer (13) found some evidence in favor of a high protein intake in his studies. Drill *et al.* (8) reported that hepatic function was as good in dogs on an 8% casein as on a 20% casein diet after carbon tetrachloride administration. Methionine had no effect when added to either diet. There is some agreement that a diet which keeps liver fat low and protein stores high will offer maximal protection against injury by hepatotoxins. Once the liver has been badly injured the best dietary management is less clear. During the period of nitrogen loss it is difficult to prevent a negative balance by increased protein intake and it has been questioned whether it is wise to try (29). If a protein anabolic phase occurs during rapid regeneration it would seem logical to feed extra protein. Ravdin *et al.* (30) favor liver as a major source of protein during recovery, but Davis and Whipple (7) found bread and skim milk equally effective.

Between the lowest level of protein intake which will maintain nitrogen balance and protein stores, and relatively high levels, there is a broad range in which it is difficult to demonstrate any difference in the ability of the liver to withstand a single exposure to hepatotoxin or to effect rapid repair. Evidence already cited suggests that this wide range of protein intake may be narrowed from each end by different factors. Continued administration of a toxic agent will cause nitrogen loss and increase ultimately the requirement for protein in the diet. Gross interference with the natural state of the liver, by formation of an Eck fistula, ligation of the bile duct, or the production of severe injury, is known to reduce drastically the amount of protein in the form of meat that the animal can be fed with impunity.

Two important points remain to be settled in this connection. The "high protein" diets that were harmful to dogs with experimentally deranged livers, were lean meat diets. The effects in most cases could be produced by protein-free extractives of the meat. Bollman has been quoted (10) and Johnson *et al.* (17) state that protein in large amounts from nonmeat sources is not harmful under these conditions, but this has not been reported in a well controlled study. One must also consider possible counterparts of the animal experiments in human liver disease. So far as we can ascertain no instance of a similar effect in patients with prolonged obstructive jaundice or advanced cirrhosis has been described and attributed to a high meat diet. Morrison (27) reports, but does not discuss, the case of two patients without ascites who became bedridden with this condition when placed on a high protein, high meat intake. A survey of the literature suggests that in patients with severe liver disease there are those who will tolerate a lot of protein, even in the form of meat, and appear to derive benefit from it. There are others who will show no improvement on a high protein regimen and, if meat is a major source of this protein, there are some who will deteriorate more rapidly. It would be helpful if one could distinguish between these cases.

Our series of animals was admittedly small, but the conditions were carefully controlled and the observations covered in several of the dogs nearly a quarter of their natural life span. The data presented has led us to make the following deductions. Animals on a restricted protein intake showed less disturbance of hepatic function during the acute phase of carbon tetrachloride administration. A somewhat more rapid restoration of normal function was observed when the dogs recovered on the high protein intake. The serum protein pattern was altered to about the same extent on either diet during the acute phase. In the recovery period the ratio tended to return toward normal more rapidly when the high protein diet was fed.

In so far as these observations can be applied to humans they lend support to the view that the feeding of large amounts of protein (at least in the form of meat) is undesirable in the presence of acute liver damage. A patient with hepatitis is unlikely, without persuasion, to eat much meat during the acute phase of the illness because of anorexia. There would seem to be no cause for alarm if the protein intake at such a time is only 25–50 gm. daily, provided

adequate carbohydrate is supplied. Once the acute phase has passed, an event which may be signalled by return of appetite, a generous amount of protein (100–125 gm.) in a balanced diet probably offers optimal nutrition for repair of the liver. In far advanced chronic disease of the liver a generous intake of protein is likely to be well tolerated and may be beneficial, but the evidence that diets containing 140, 175, or 300 gm. of protein are superior is not impressive.

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A METHOD FOR THE QUANTITATIVE MEASUREMENT OF AGGLUTININ NITROGEN IN ANTISERA TO *HEMOPHILUS PERTUSSIS*, PHASE I[1]

By Catherine F. C. MacPherson,[2] Paul H. Maurer,[3,4] Hattie E. Alexander,[3] and Winifred Redman[3]

Abstract

A method for the measurement, in weight units, of the agglutinin nitrogen in antisera to *Hemophilus pertussis*, Phase I, is described. The procedure and techniques are essentially the same as those first published by Heidelberger and Kabat for the measurement of agglutinin nitrogen in antipneumococcal sera. However, it was found that unusually large amounts of bacterial nitrogen were required to absorb pertussis agglutinins completely, even when the antibody content of the antisera was low. The accuracy of this method is limited to ± 1-2%, the error involved in the Kjeldahl procedure for measuring nitrogen. Thus, the large amounts of bacterial nitrogen necessary for absorption and the relatively low agglutinin nitrogen content of the antisera indicated that the results of one, or at the most two, absorptions be used to calculate values with some meaning in terms of quantitative analytical chemistry. Analysis of an unknown antiserum can now be accomplished in one or two absorptions if 1.0 ml. volumes of appropriate dilutions of serum are absorbed with approximately 2 mgm. of bacterial nitrogen. The antibody content is calculated from the dilution of serum which was completely absorbed.

Introduction

The need for a quantitative measure of antibody content as an essential part of the evaluation of the therapeutic efficacy of an antiserum is now amply shown (8). Until now the strength of *H. pertussis* antisera has been estimated by their relative agglutinin titers and by assay of the protective power for mice. Both methods are subject to large errors, are uncertain, and neither gives information as to the actual amounts of antibody involved (14). Similar considerations led to the quantitative agglutinin nitrogen method of Heidelberger and Kabat for measuring antibody in antipneumococcus antiserum (5) and later Alexander and Heidelberger in *H. influenzae* rabbit antiserum (1). The present paper describes an adaptation of this procedure to the measurement in weight units of agglutinin nitrogen in *H. pertussis* antisera.

Materials and Methods

Serum H1 was a pool of sera from 30 normal infants, three to six months old, who had had no history of whooping cough and no artificial immunization.

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[2] *Research Fellow, 1947-1949, Department of Bacteriology and Immunology, McGill University, Montreal, Que. Present address, 236 Brock Ave. N., Montreal West, Que.*

[3] *Department of Pediatrics, College of Physicians and Surgeons, Columbia University, New York, N.Y.*

[4] *Present address, University of Pittsburgh, School of Medicine, Pittsburgh, Penn.*

These sera were obtained through the courtesy of the Child Health Association of Montreal. Serum H2 was a pool from five children about five years old. H3 was a pooled lot from the sera of eight normal adults. CB was a commercial preparation of the globulin fraction of sera from humans immunized with *H. pertussis*, Phase I, and represents a 10-fold concentration of serum. P1 was a commercial product in lyophilized form prepared from 20 ml. of serum from immunized human adults. For this study it was reconstituted to one-half its original volume. HG was a sample of human gamma globulin prepared from the blood of normal adults according to the methods of Cohen *et al.* (2) and Oncley *et al.* (12). New York No. 27, obtained from the New York State Department of Health, was an antipertussis rabbit serum concentrate. NR was a pool from the sera of several normal rabbits. M No. 57 was an unconcentrated rabbit antiserum produced by the Michigan State Department of Health.

Bacterial Suspensions

A single Phase I strain of *H. pertussis* No. 18323-M, supplied by Dr. Pearl Kendrick, was used for all bacterial suspensions employed in measuring agglutinin nitrogen. Starting with a culture preserved and sealed under vacuum the desiccated organisms were suspended in nutrient broth by adding 0.2 ml. to the tube in which the culture was dried, and this suspension was used to seed the surface of a Bordet–Gengou agar (Difco) plate. After 48 hr. incubation the growth was transferred to 1 ml. of broth and seeded in 0.5 ml. quantities on each of two Bordet–Gengou agar plates; the growth from each following incubation for 24 hr. was suspended in 5 ml. of broth; 0.5 ml. quantities were seeded on each of 10 Bordet–Gengou agar plates. The growth after 24 hr. was removed from each of these plates with 5 ml. of neopeptone broth. The suspensions from the 10 plates were combined and 0.5 ml. quantities were seeded on each of 100 plates of Bordet–Gengou agar. To the growth obtained on each plate in 24 hr. was added 5 ml. of 0.2% of formalin in saline and the growth was removed by gently scraping the surface with a smooth tip of a pipette. The yield from 100 plates was centrifuged in the cold at 3000 r.p.m., the supernatant was decanted, and the cells were washed twice in 0.2% of formalin in saline. Just prior to use for quantitative serological analysis the formalinized bacterial suspensions were washed with 0.85% saline, first at 37° C., and then at 0° C., until supernatants contained less than 0.01 mgm. N per ml. This washing procedure simulated the washing conditions after agglutination. Usually three to five washings were sufficient. The washed bacteria were smoothed out with a stirring rod to break up clumps. Saline was added to make suspensions at two concentrations containing about 0.4 mgm. and 1.0 mgm. bacterial N per milliliter. Each suspension was filtered through a loose cotton plug to remove residual clumps of bacteria and lumps of agar and merthiolate added to a concentration of 1 : 10,000.

Stability of Bacterial Suspensions and Specificity of Agglutinin Nitrogen

Vaccines to be used for quantitative analyses were tested for their stability in buffered saline and in normal rabbit and normal human sera buffered at pH 7.5, 6.9, and 6.4 (16). Two milliliters of *M*/15 phosphate buffer were added to accurately measured volumes of vaccine at 0° C. After thorough mixing, the suspensions were allowed to stand overnight at 0-5° C., centrifuged in the cold, and washed twice at 0° C. with 3.0 ml. of buffered saline. The N content of the washed suspensions was determined by the Markham micro-Kjeldahl method (7). The results, given in Table I, Column 3, are typical

TABLE I

EFFECT OF pH AND SALINE WASHINGS ON THE CELLULAR N OF *H. pertussis* STRAIN NO. 18323-M GROWN ON BORDET–GENGOU AGAR AND ON THE AMOUNTS OF N THESE VACCINES REMOVED FROM POOLED NORMAL HUMAN AND RABBIT SERA

Serum	pH of cell–saline or cell–serum mixtures	N recovered from cell–saline mixtures after washing, mgm.	N recovered from cell–serum mixtures after washing, mgm.	N absorbed from 1.0 ml. 1:1 dilution, mgm.
H1	6.9	1.14	1.14	0.00
H2	7.5	0.60	0.62	0.02
	6.9	0.58	0.62	0.04
	6.4	0.59	0.62	0.03
H3	7.5	0.49	0.51	0.02
	6.9	0.49	0.52	0.03
	6.4	0.50	0.52	0.02
NR	6.9	0.82	0.84	0.02

NOTE: *H1, Pool of sera from 30 normal infants ages three to six months.*
H2, " " " " five " children age five years.
H3, " " " " eight " adults.
NR, " " " " normal rabbits.

of several sets of analyses, and show that the vaccines were stable at each pH tested when exposed to conditions necessary for thorough washing after agglutination. About 0.04 mgm. N per mgm. of bacterial N was lost during the washing process. Some of this has been found to be protein N (10, 3). Although the antigenic components of the surface of *H. pertussis* responsible for agglutinin formation are protein in nature (14, 15), it has been shown that the washing process (10, 3) made no difference either in the agglutinability or in the capacity of washed bacteria to produce good mouse protection antisera (11).

Pooled normal human sera were diluted 1 : 1 or 2 : 3 with *M*/15 phosphate buffers at pH 7.5, 6.9, and 6.4. After standing overnight in the cold, and centrifugation, 1.0 ml. aliquots of buffered sera were added to accurately measured volumes of similarly buffered vaccine. Control suspensions contained the same amounts of vaccine but were adjusted with buffer to the same volume as the mixtures containing serum. The mixtures were left overnight

in the cold and then centrifuged and washed with buffered saline. It will be noted in Table I that similar amounts of N were removed from "normal" human sera at each pH tested, with a possible optimum at pH 6.9. These sera and normal rabbit sera always agglutinated the vaccines. Not until the pool of sera from infants three to six months of age was studied at pH 6.9 could it be certain that the method measured only specific N at pH 6.9. This serum did not agglutinate our vaccines, and no N was removed from it when quantitative absorptions were carried out at 0° C. or 37° C. In addition, slide and tube agglutination tests, performed in the standard way and using other Phase I strains, were negative. Based on these studies and the desire to approach neutrality all serum analyses were carried out at pH 6.9.

Agglutinin Nitrogen

An accurately measured amount of a washed bacterial suspension is added to an accurately measured volume of antiserum, the relative amounts being so chosen that the bacteria are in excess. After incubation of 37° C. for three hours and 0–5° C. for 16 hr., or for 20 hr. at 0° C., the difference between the nitrogen content of the agglutinated bacilli, suitably washed (see below), and that of the same quantity of unagglutinated washed bacilli gives the agglutinin nitrogen removed during each absorption from the volume of serum chosen. The supernatant is tested to make certain that all antibody has been removed; if some antibody remains, absorption on a fresh suspension is repeated. As will be shown later the best results are obtained when the agglutinins are removed from 1 ml. of diluted antiserum with a charge of 2 mgm. of bacterial N.

For hyperimmune human serum or serum concentrates usually 1 or 2 ml. of serum were pipetted into 5 ml. volumetric flasks containing a drop of 0.04% phenol red and $N/10$ hydrochloric acid was added dropwise until the color just changed to orange. Then $M/15$ phosphate buffer, pH 6.9, was added to volume, and the contents of the flask were well mixed and refrigerated overnight. Immune rabbit sera were similarly diluted 1 : 4 or 1 : 9 depending on the strength of the sera. When sera contained precipitable antibodies to the Bordet–Gengou medium, these were removed by adding a sufficient amount (determined by preliminary tests) of a saline extract of Bordet–Gengou media before diluting to volume with buffer. In the early analyses 1 ml. of diluted serum was added to 1.5 to 2.0 ml. aliquots of vaccine and mixed with 1 ml. of $M/15$ phosphate buffer at pH 6.9 in 15 ml. conical centrifuge tubes. Control suspensions contained the same amount of vaccine and the buffer instead of serum. The tubes were well mixed every 15 min. for three hours either at 37° C. or 0° C. and then refrigerated overnight. The tubes were spun in the cold at 3000 r.p.m. for one hour and the resulting precipitates washed (5) twice with 3.0 ml. of 0.85% saline at 0° C. *H. pertussis* suspensions centrifuged to a compact mass and were almost impossible to resuspend evenly for thorough washing. This difficulty was first overcome by adding a glass bead to each tube just before centrifugation. After the supernatant was poured off, the clump could be completely broken

up by rotating the glass bead. This process was abandoned because it was tedious and splashing sometimes occurred. The method now used depends upon diffusion of serum out of the clump. When tubes have been drained, usually 0.1-0.2 ml. of supernatant remains. By allowing drained tubes to stand for one-half hour in an ice bath, the clumps could be loosened from the bottom by twirling the tubes a few times. After the addition of 2 to 3 ml. of cold saline the clump is made to travel up through the saline several times over the course of one-half hour by twirling the tubes. Quantitative comparisons of the two methods of washing the agglutinated bacilli yielded the same results.

After the first absorption, supernatants of duplicates were usually combined for subsequent absorptions unless the sera were very strong. Absorptions were continued until the supernatants did not agglutinate fresh charges of vaccine.

From the analyses summarized in Tables II and IV and a number of other analyses not given, it is apparent that the conditions found adequate for the measurement of agglutinin nitrogen in nontypable pneumococcal or influenzal antisera (5, 9) are unsatisfactory for the study of *H. pertussis* antisera. While several absorptions are necessary to remove somatic agglutinins from anti-pneumococcal or anti-influenzal sera, the total agglutinin content and the fraction of antibody removed in the early absorptions were great enough so that the error inherent in the analysis for N was an acceptable percentage of the total agglutinin content. In diluted human *H. pertussis* antisera the amount of antibody measured at each absorption is not greater than the error of the Kjeldahl method. In stronger sera where four to six absorptions are necessary to remove all antibody, the summation of technical errors makes calculation of antibody content quantitatively meaningless.

An attempt was made to solve these difficulties by reducing the number of absorptions needed by increasing the efficiency of each. Three factors were explored to determine their effect on the efficiency of absorption of agglutinin nitrogen by bacterial suspensions: (1) Temperature at which absorptions are carried out, (2) Dilution of serum absorbed, (3) Quantity of bacterial nitrogen used.

Influence of Temperature and Dilution

Tables II and IV list the results of analyses carried out on rabbit serum New York State No. 27 when diluted 1 : 1 at 0-5° C. for 20 hr. and for comparison the results obtained from analyses carried out at 37° C. for three hours and 16 hr. at 0-5° C. and on Michigan No. 57 at various dilutions. Absorption at the higher temperature resulted in somewhat more efficient removal of agglutinin in the first and second absorption as noted by Heidelberger and Kendall (6). Under similar conditions the serum diluted 1 : 9 shows further increase in antibody removed.

The results of analyses of CB, human serum globulin, run at 0-5° C. and 37° C. for three hours are shown for comparison.

TABLE II

INFLUENCE OF TEMPERATURE AND DILUTION ON EFFICACY OF ABSORPTION OF AGGLUTININ NITROGEN

Serum	Serum volume, ml.	Absorption 1			Absorption 2			Absorption 3			Absorptions 4 and 5	Dilution	Total N absorbed per ml. undiluted, mgm.
		Vaccine N added, mgm.	N found, mgm.	N absorbed, mgm.	Vaccine N added. mgm.	N found, mgm.	N absorbed, mgm.	Vaccine N added, mgm.	N found, mgm.	N absorbed, mgm.	Total N, mgm.		
		20 hr., 0°-5° C.			48 hr., 0°-5° C.			20 hr., 0°-5° C.					
H1	1.0	1.14	1.14	0.00									0.00
CB	1.0	0.49	0.55	0.06	0.50	0.55	0.025	0.49	0.55	0.03		2 : 5	0.29
N.Y. No. 27	1.0	1.08	1.43	0.35	1.11	1.28	0.17	0.81	0.99	0.18	0.26	1 : 1	1.92
		37° C., 3 hr.; 0°-5° C. 16 hr.			20 hr., 0°-5° C.			37° C., 3 hr.; 0°-5° C. 16 hr.			20 hr., 0°-5° C.		
N.Y. No. 27	1.0	0.82	1.32	0.48	0.81	1.09	0.28	0.68	0.88	0.20	0.19	1 : 1	2.30
		All absorptions: 37° C., 3 hr.; 0°-5° C., 16 hr.											
N.Y. No. 27	1.0	0.56	0.70	0.14	0.73	0.83	0.05	0.73	0.78	0.03	0.73, 0.77, 0.04	1 : 9	2.60
H1	1.0	0.56	0.55	0.00	0.73	0.74	0.01					2 : 3	0.00
HG	1.0	0.56	0.59	0.03	0.73	0.78	0.03	0.73	0.76	0.02		1 : 4	0.45

TABLE III

INFLUENCE OF QUANTITY OF BACTERIAL NITROGEN IN EFFICIENCY OF ABSORPTIONS

Serum	Serum volume, ml.	Absorption 1			Absorption 2			Absorption 3			Total N absorbed per ml. serum, mgm.	Temperature	Dilution
		Vaccine N added, mgm.	N found, mgm.	N absorbed, mgm.	Vaccine N added, mgm.	N found, mgm.	N absorbed, mgm.	Vaccine N added, mgm.	N found, mgm.	N absorbed, mgm.			
Pl	1.0	0.49	0.53	0.04	0.50	0.52	0.01	0.49	0.51	0.01	0.12	0°- 5° C., 20 hr.	1 : 1
Pl	1.0	2.00	2.14	0.14	No agglutination by supernatant						0.14	37° C., 3 hr.; 0°-5° C., 16 hr.	Undiluted
HG	1.0	0.56	0.59	0.03	0.73	0.78	0.02$_5$	0.73	0.76	0.01$_5$	0.35	37° C., 3 hr.; 0°-5° C., 16 hr.	1 : 4
HG	2.5	2.00	2.15	0.15	No agglutination by supernatant						0.30	37° C., 3 hr.; 0°-5° C., 16 hr.	1 : 4
H3	1.0	0.49	0.52	0.03	Only one absorption						0.08	0°-5° C., 20 hr.	2 : 5
H3	1.0	2.00	2.08	0.08	No agglutination by supernatant						0.08	37° C., 3 hr.; 0°-5° C., 16 hr.	Undiluted

TABLE IV

INFLUENCE OF DILUTION OF MICHIGAN NO. 57 ANTISERUM AND CONCENTRATION OF BACTERIAL N ON EFFICIENCY OF ABSORPTION OF AGGLUTININ N; 37° C., 3 HR.; 16 HR., 0°-5° C.

Serum volume, ml.	Absorption 1			Absorption 2			Absorption 3			Total N absorbed per ml. serum, mgm.	Dilution	N absorbed per ml. undiluted serum, mgm.
	Vaccine N added, mgm.	N found, mgm.	N absorbed, mgm.	Vaccine N added, mgm.	N found, mgm.	N absorbed, mgm.	Vaccine N added, mgm.	N found, mgm.	N absorbed, mgm.			
1.0	1.90	2.11	0.21	1.91	1.98	0.07	1.89	1.97	0.08*	0.32	Undiluted	0.32
1.0	1.90	1.99	0.09	1.91	1.91	0.00*	1.89	1.96	0.07*	0.13	1 : 4	0.65
1.0	1.90	1.96	0.06	1.91	1.92	0.01*	1.89	1.92	0.03*	0.08	1 : 9	0.80
Diluted vaccine												
1.0	0.368	0.470	0.102	0.368	0.394	0.026	0.380	0.390	0.010*	0.133	Undiluted	0.133
1.0	0.368	0.416	0.048	0.368	0.366	0.00	0.380	0.408	0.028*	0.062	1 : 4	0.310
1.0	0.368	0.404	0.036	0.368	0.398	0.030*	0.380	0.388	0.008*	0.055	1 : 9	0.550

* *Analyses run on pooled supernatants.*

The results of analyses of normal infant serum H1 carried out at 37° C. are included. That no measurable agglutinin nitrogen was found, even under conditions which permit more efficient absorption of antibody, demonstrates again the fact that the method measures only antibody specific for *H. pertussis*.

Influence of Quantity of Bacterial Nitrogen

When unconcentrated human antisera are absorbed with the largest quantity of bacterial nitrogen permitting accuracy (2 mgm.) at 37° C. for three hours, followed by 16 hr. at 0-5° C., virtually all measurable antibody is removed by one absorption. This is shown in Table III.

The results show that, when the bacterial nitrogen used for absorption is increased to 2.0 mgm. for absorption of each milliliter of serum measured, the efficiency of the first absorption is greatly increased. This is true for human hyperimmune and rabbit antisera.

The data presented in Tables III and IV suggest that the agglutinin content of both human and rabbit *H. pertussis* antisera can be measured most accurately and in the shortest time by using a large enough number of dilutions of an unknown serum in order to include that dilution which permits complete removal of the antibody by one absorption,—1 : 1, 1 : 5, and 1 : 10 dilutions should meet the requirements of human and rabbit antisera. They suggest further that 2.0 mgm. of bacterial nitrogen per milliliter of serum absorbed at 37° C. for three hours and 0-5° C. for 16 hr. provide the optimal conditions for the most efficient absorption.

Discussion

The method for measuring agglutinin nitrogen herein described has difficulties and limitations. However it is reproducible. The difficulties in determining the agglutinins to *H. pertussis* are encountered in (1) preparing a suitable vaccine, i.e., one which centrifuges to a compact mass with very little soluble nitrogenous material remaining in the supernatant and (2) keeping the agglutinated bacteria continually suspended for at least three hours so as to permit maximal absorption of antibody. The main limitation to the method is that large amounts of bacterial N are required to absorb small amounts of antibody. Since the maximal amount of bacterial N which this technique permits is 2.0 mgm. the accuracy is limited to $\pm$ 0.02 to 0.04 mgm. of antibody N (7). Therefore one has to use that dilution of serum which will allow the antibody to be removed in at most two absorptions. Thus the value obtained with 2 mgm. bacterial N and that dilution of serum which allows agglutinins to be removed completely by the second absorption is the one to be preferred.

At present there is no good biological or chemical test to confirm or refute the quantitative values. Mouse protection tests done in conjunction with Dr. Pearl Kendrick on unabsorbed and both partially and completely absorbed rabbit and human sera have not led to any correlations between the concentration of agglutinin N and the protective power of these sera. Further

work is contemplated to compare the antibody N value obtained with whole bacteria and the purified protein fractions (4, 13) responsible for the formation of agglutinins.

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